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These studies demonstrate that lipid composition of both diatom cultures and natural phytoplankton assemblages varies greatly on a diel cycle. Exposure experiments on both cultures of diatoms and natural assemblages demonstrated that different results could be obtained by merely changing the timing of the initial exposure. Cells that were undergoing changes in lipid composition appeared to be most susceptible to chlorinated hydrocarbons, especially when periods of polar lipid synthesis immediately followed the exposure. However, as a rule, the chlorinated benzenes at concentrations approaching water solubility, did not appear to have significant long term effects on the diatom cultures studied. Short-term changes were observed, but recovery was also evident. The effect of timing of exposure suggests that standard toxicity tests should be conducted with extreme care since results may vary with the natural diel lipid cycle.

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# INFLUENCE OF LIPID COMPOSITION IN AMPLIFYING OR AMELIORATING TOXICANT EFFECTS ON PHYTOPLANKTON

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**Abstract.** These studies demonstrate that lipid composition of both diatom cultures and natural phytoplankton assemblages varies greatly on a diel cycle. Exposure experiments on both cultures of diatoms and natural assemblages demonstrated that different results could be obtained by merely changing the timing of the initial exposure. Cells that were undergoing changes in lipid composition appeared to be most susceptible to chlorinated hydrocarbons, especially when periods of polar lipid synthesis immediately followed the exposure. However, as a rule, the chlorinated benzenes at concentrations approaching water solubility, did not appear to have significant long term effects on the diatom cultures studied. Short-term changes were observed, but recovery was also evident. The effect of timing of exposure suggests that standard toxicity tests should be conducted with extreme care since results may vary with the natural diel lipid cycle.

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## Summary

Previous work in our laboratory (Sicko-Goad et al. 1988; 1989a,b,c,d) suggested that in the diatom, *Cyclotella meneghiniana*, lipids increased when cells were exposed to chlorinated benzenes and that there was an increase in total lipid during the dark phase of the daily photoperiod. Similar increases in lipid content upon exposure to toxicants had been demonstrated by Soto et al. (1977) and Fisher et al. (1976). These observations suggested that certain algal populations might be more susceptible to partitioning of lipophilic toxicants due to increases in lipid content as a result of environmental variables such as photoperiod, nutrient limitation, or pre-exposure to other toxicants.

Our work further suggested that the timing of the initial exposure to lipophilic toxicants produced quite different results than would be predicted by partition coefficients alone. That is, the most highly lipophilic chlorinated benzene that we tested produced the least number of changes in cellular parameters measured throughout the exposure experiment. We found that octanol-water partition coefficients alone were not accurate predictors of toxicity at concentrations found in the aquatic environment. Rather, toxicity appeared to be related more to chemical reactivity and the amount of internal lipid pools (Sicko-Goad et al. 1989a,b,c,d). For example, trichlorobenzenes with more vicinal carbon atoms were more toxic to phytoplankton, but predicted sensitivity to the compounds (based on reactivity) was altered when algal cells containing more lipid were exposed to toxicants.

This led us to hypothesize that some algal populations might be more susceptible to partitioning of lipophilic compounds when they were grown under sub-optimal conditions such as nitrate limitation (which promotes lipid production) or when they had been pre-exposed to other toxicants, either inorganic or organic. We further hypothesized that the timing of the exposure, both in reference to the time of day and the stage of growth were important factors in determining toxicity effects.

We therefore proposed to study the relationship between lipid composition and toxicity, with the following objectives:

1. To determine the relationship between growth phase and toxicity,
2. To determine the relationship between lipid composition and toxicity,
3. To determine if lipid rich cells (i.e. senescent cells) exposed to toxicants experienced detrimental effects upon resumption of active growth.

## Introduction

This final technical report is divided into three broad sections:

A. Published manuscripts or manuscripts in press, and in review;

B. Full copies of papers presented at scientific meetings where the data has not yet been formally rewritten for peer-reviewed journals, and

C. Status of unpublished data

A1. ***Effects of chemical fixation on the flow cytometric properties of diatoms.*** Paper presented at the 11th International symposium on living and fossil diatoms. Full-length manuscript, *In Press*, Proceedings of the International Diatom Conference, California Academy of Science.

A2. ***Effect of growth and light/dark cycles on diatom lipid content and composition.*** Sicko-Goad, L., and N. A. Andresen. 1991. J. Phycology 27:710-718.

A3. ***Effect of diatom lipid composition on the toxocity of chlorinated benzenes. I. Short-term effects of 1,3,5-trichlorobenzene.*** Submitted to Archives of Environmental Contamination and Toxicology. 1992.

A4. ***Effect of diatom lipid composition on the toxocity of chlorinated benzenes. II. Long-term effects of 1,2,3-trichlorobenzene.*** Submitted to Archives of Environmental Contamination and Toxicology. 1992.

B1. ***Periodicity of lipid content and composition in diatoms.*** Presented at 1990 meeting of the Phycological Society of America.

B2. ***Photoperiod and temperature effects on diatom growth.*** Presented at the 1991 meeting of the American Society of Limnology and Oceanography.

B3. ***Photoperiod and temperature effects on diatom lipid composition. I. Cyclotella meneghiniana.*** Presented at the 1991 meeting of the International Phycological Society.

B4. ***Photoperiod and temperature effects on diatom lipid composition. II. Melosira varians.*** Presented at the 1991 meeting of the International Phycological Society.

## A1 –

# EFFECTS OF CHEMICAL FIXATION
# ON THE FLOW CYTOMETRIC PROPERTIES OF DIATOMS

## ABBREVIATIONS:

| | |
|---|---|
| BFL: | linear blue fluorescence (DNA content) |
| Chl: | chlorophyll |
| LFALS: | logarithmic forward angle light scatter |
| LGFL: | logarithmic green fluorescence |
| lin: | linear |
| LOFL: | logarithmic green orange fluorescence |
| log: | logarithmic |
| LRFL: | logarithmic red fluorescence |
| L90LS: | logarithmic ninety degree light scatter |
| PE: | phycoerythrin |
| 1 P | 1 parameter |
| 2 P | 2 parameter |


## ABSTRACT

We have utilized flow cytometry to assess the effects of fixation and storage on light scatter and autofluorescence values for three diatoms: *Thalassiosira weisflogii*, *Stephanodiscus binderanus* and *Cyclotella meneghiniana*. We have also measured autofluorescence and DNA content changes in a culture of *C. meneghiniana* over a 24 hour period. Cultures of the diatoms were fixed in 1% paraformaldehyde, 1% glutaraldehyde and .05M cacodylate buffer. Measurements of light scatter and autofluorescence were made with an EPICS C flow cytometer (Coulter Cytometry) using 200 mW of 488 nm excitation. Emissions were measured in the red and green-orange regions to assess fluorescence from chlorophyll. Measurements of fixed cells were compared to those of unfixed cells from the same culture. Cells were stored in the dark at 4°C for a period of 1 month, and measurements were taken several times over that period. Light scatter and fluorescence values were unaffected by fixation, but red fluorescence values dropped significantly during the storage period for *T. weisflogii*, and to a lesser extent, for *S. binderanus* in both fixed and unfixed samples. *C. meneghiniana* maintained its original scatter and fluorescence characteristics over the storage period. Duplicate samples taken every 2 hours from an unsynchronized, logarithmically growing culture of *C. meneghiniana* grown on a 16:8 L/D cycle at 15°C were fixed and stored as above for 2 weeks. Samples were then stained with the DNA-specific dye Hoechst 33342 at 5 μg/ml for 1 hour in the dark at room temperature. Two populations were identified by correlating DNA and chlorophyll content in a 2 parameter histogram display: cells in one group contained 6 times the red fluorescence (Chl) and 1.7 times the DNA content, on average, as cells in the second group. A small, but significant, drop in the number of cells with high DNA and Chl content occurred between 1500 and 1900 hours (hours 5 to 9 of the light period), and returned to original levels by 2300 hours.



Key Words: *Thalassiosira*, *Stephanodiscus*, *Cyclotella*, flow cytometry, fixation

## INTRODUCTION

Flow cytometry has been used by many investigators to analyze characteristics of phytoplankton populations, including relative cell size and shape, the fluorescence emissions of endogenous or exogenously added fluorochromes, and discrimination of dead cells, debris and bacterial contamination (e.g., Olson *et al.* 1983, Chisholm *et al.* 1988, Olson *et al.* 1989, Robertson and Button 1989, Premazzi *et al.* 1989, Phinney and Cucci 1989). Many investigators do not have immediate access to a flow cytometer and must store samples for days before analysis can be performed. We have studied the effects of fixation and storage on light scatter and autofluorescence emissions of three cultured diatoms, and subsequently measured autofluorescence and DNA content changes in fixed samples taken over a 24 hour period from a culture of log phase *Cyclotella meneghiniana*.

## MATERIALS AND METHODS

For fixation experiments, 1 to 2 x $10^6$ cells were taken from unsynchronized stock cultures of *Cyclotella meneghiniana, Stephanodiscus binderanus* and *Thalassiosira weisflogii*.

Cells were pelleted by centrifugation, washed once in medium, and resuspended in either medium or a fixative solution containing 1% paraformaldehyde, 1% glutaraldehyde, and 0.05 M cacodylate buffer, pH 7.2. Samples were stored at 4°C in the dark. Duplicate samples were also taken every 2 hours over a 24 hour period from an unsynchronized, logarithmically growing culture of *C. meneghiniana* grown on a 16:8 L/D cycle at 15°C. Cells were fixed as above and stored for 2 weeks at 4°C in the dark.

For DNA staining, 5 x $10^5$ cells were pelleted and resuspended in a 5 μg/ml solution of Hoechst 33342 (Sigma, St. Louis, MO) for 1 hour in the dark at room temperature.

**Flow Cytometry**. All measurements were made with an EPICS C flow cytometer (Coulter Cytometry, Hialeah, FL) operated at either the blue 488 nm excitation line (200 mW) or the ultraviolet 364 nm line (50 mW) of an argon laser. Autofluorescence measurements were made in both the red (using a 630 longpass filter) and the green-orange (using the combination of a 590 dichroic mirror and 515 laser blocking filter) regions, and were collected with logarithmic amplifiers. Hoechst 33342 fluorescence (364 nm excitation) was collected linearly using a 440 bandpass filter. Forward and ninety degree angle light scatter were collected logarithmically, and used for gating as described in Results. Fluorescence and light scatter values for diatom samples run on different days can be directly compared because (i) identical photomultiplier tube and laser settings were used on all days, and (ii) a fluorescent bead standard (Immunocheck Beads, Coulter) was used to align and calibrate the instrument to give identical fluorescence and scatter readings each day. Data was collected as either two parameter (2P) 64 x 64 channel or one parameter (1P) 256 channel histograms. Mean logarithmic fluorescence values were converted to mean linear values to facilitate data comparisons using the method of Muirhead *et al.* (1983).

## RESULTS

An examination of the 2P log forward and log ninety degree light scatter plots (LFALS and L90LS, respectively) collected 1 hour after fixation showed that all three cultures contained at least two distinct populations of particles (Fig. 1 a,b,c). Further analysis of these populations showed that only the group with high LFALS values (designated in Figure 1 by the boxed area) contained cells with substantial red autofluorescence (LRFL) emissions. Sorting of these two populations confirmed the high LFALS group to be whole diatoms, while the lower population consisted of debris and cell fragments. Subsequent analysis was confined to the high LFALS group.

Several values derived from the flow histograms were used to assess fixation and storage effects over time: (i) the percentage of cells with "whole cell" light scatter characteristics (i.e., high LFALS and L90LS values as shown in Fig. 1), (ii) the percent of these cells with high LRFL values, defined as marked for each diatom in Fig. 1d,e,f (cells sorted from populations with red fluorescence values below this level were judged microscopically to be non-viable), and (iii) the mean LRFL and log green-orange fluorescence (LOFL) values of these "bright" red autofluorescent cells. These values are presented in Table I.

Changes in these parameters occurred over time in two of the three tested diatom species, but appeared to be independent of fixation (Fig. 2, Table I). Neither fixation nor storage (at 4°C in the dark) had any measurable affect on the percent of cells with "whole cell" scatter characteristics; these values varied greatly between the cultures, however, with *T. weisflogii* having the lowest number of whole cells (5-8%) and *S. binderanus* having the greatest (51-57%). Variation seen in light scatter patterns between the species is probably due to their morphologic differences (Trask *et al*. 1982, Olson *et al*. 1989, Premazzi *et al*. 1989).

The percentage of bright red autofluorescent cells (defined as marked in Fig. 1) was not affected by fixation, but dropped over the storage period for *T. weisflogii* (from 75% to 42% for unfixed, and 78% to 51% for fixed cells), and to a lesser extent, for *S. binderanus* (77% to 60% unfixed, 76% to 60% fixed). The most dramatic drop in these values occurred in the first 24 hours of storage. *C. meneghiniana* samples were the most stable, maintaining values of 27% to 33% of cells with bright red autofluorescence.

The changes observed in mean LRFL and mean LOFL values of the bright red fluorescing cells were not significant for any of the cultures given the broad fluorescence distributions (i.e., large coefficients of variation) of the histograms. However, it is interesting to note the relative differences between diatom species in terms of these values: a comparison of mean linear fluorescence values showed *C. meneghiniana* and *T. weisflogii* to have 3 to 5 times the red chlorophyll fluorescence of *S. binderanus* (compare linear fluorescence values in Table I).

DNA content and red autofluorescence were measured simultaneously on fixed samples of *C. meneghiniana* taken over a 24 hour period and stained with the DNA-specific dye, Hoechst 33342. We have found that 364 nm excitation of chlorophyll (which occurs either directly or through energy transfer by accessory pigments), while not a peak excitation wavelength, is sufficient to produce a strong, measurable emission above 630 nm. A comparison of *C. meneghiniana* red fluorescence distributions collected with 488 nm and then 364 nm, excitation showed the percentage of red fluorescing cells and their distributions to be essentially the same (data not shown). A representative 2 parameter plot of Hoechst stained cells is presented in Figure 3, along with a plot of unstained cells from the same sample. It was apparent from 1P histograms that the cells with LRFL values off scale negative in unstained samples increased their LRFL values upon Hoechst staining, while fluorescence emissions of cells with high LRFL values were unaffected by the stain (Fig. 3). Further analysis was confined to those cells whose LRFL values were unaffected by Hoechst staining because sorting and microscopic examination of cells with off-scale negative LRFL values showed these to be whole cells with little or no autofluorescence, and were designated as "dead" for the purposes of our analysis. We attribute the increase in LRFL emissions of these cells to secondary excitation of chlorophyll by the blue fluorescence emissions of Hoechst stain taken up non-specifically (Trask *et al*. 1982, Loken 1980, Premazzi *et al*. 1989).

Two populations were identified by their chlorophyll and DNA contents, and the numbers of cells in each determined (see Figure 3, boxed areas). The cells in Box 1, Figure 3, had 6.0 times the red fluorescence and 1.7 times the Hoechst-DNA fluorescence of cells in Box 2. The percentage of cells in Box 1 was determined for each time point and plotted (Fig. 4). A small, but significant, drop in the number of cells in this population occurred between 1500 and 1900 hours (hours 5 to 9 of the light cycle), and then increased back to its original value by 2300 hours.

Table 1. Effects of fixation on flow cytometric values of diatom species.

| Cell Type[1] | Fix | Day | % in Scatter Gate[2] | % Red Auto-fluorescent[3] | LRFL (lin)[4] | LOFL (lin)[5] |
|---|---|---|---|---|---|---|
| TW | N | 1 | 6 | 75 | 194 (59) | 187 (48) |
| | N | 2 | 5 | 51 | 189 (51) | 186 (46) |
| | N | 28 | 6 | 42 | 174 (32) | 184 (44) |
| TW | Y | 1 | 6 | 78 | 189 (51) | 198 (66) |
| | Y | 2 | 6 | 51 | 189 (51) | 202 (74) |
| | Y | 28 | 8 | 51 | 180 (39) | 214 (106) |
| SB | N | 1 | 51 | 77 | 125 (8) | 121 (7) |
| | N | 2 | 51 | 73 | 122 (7) | 120 (7) |
| | N | 28 | 53 | 60 | 111 (5) | 120 (7) |
| SB | Y | 1 | 53 | 76 | 123 (7) | 135 (10) |
| | Y | 2 | 57 | 83 | 125 (8) | 142 (13) |
| | Y | 28 | 51 | 60 | 115 (6) | 148 (15) |
| CM | N | 1 | 36 | 28 | 175 (33) | 162 (23) |
| | N | 2 | 36 | 27 | 173 (32) | 162 (23) |
| | N | 28 | 40 | 33 | 163 (23) | 157 (20) |
| CM | Y | 1 | 37 | 28 | 174 (32) | 179 (38) |
| | Y | 2 | 32 | | – | – |
| | Y | 28 | 31 | 27 | 167 (26) | 195 (61) |

[1] Cell Types: TW = *Thalassiosira weisflogii*
SB = *Stephanodiscus binderanus*
CM = *Cyclotella meneghiniana*

[2] Percent in Scatter Gate: Number of cells in the sample possessing "whole cell" light scatter characteristics as determined by microscope after sorting from various populations. This scatter gate is shown in Figure 1 for each cell type.

[3] Percent Red Autofluorescent: The number of cells in the scatter gate with significant red fluorescence emissions, defined as marked in Figure 1 for each species.

[4] LRFL (lin): Mean log red fluorescence value of the red autofluorescent population, and its corresponding linear value as determined by the method of Muirhead et al. (1983).

[5] LOFL (lin): Same as above, but for log green fluorescence values.

## DISCUSSION

These data show that storage of cultured diatoms in the dark at 4°C adversely effected the red autofluorescence emissions, but not the light scatter characteristics, of two out of three species studied. The fixation of samples prior to storage as described in Methods did not prevent this loss of fluorescence; in fact, fixation appeared to have no effect at all on the flow cytometric values determined for these samples. The loss of pigment fluorescence intensity with time in preserved samples is an important concern (Caron, 1983). Since flow cytometric analysis of relative fluorescence and light scatter values is being developed as a method for identifying phytoplankton species in field samples (Yentsch and Phinney 1985, Olson *et al.* 1985, 1988, 1989, Li and Wood 1988). The data presented here suggest that such identification must be made cautiously if samples are stored for even 24 hours, because not all species will undergo changes in fluorescence intensity to the same extent. Our observation that *C. meneghiniana* maintained its autofluorescence intensity under the storage conditions used here simplified further analyses because we were able to store samples until flow cytometer time was available.

Our analysis of *C. meneghiniana* sampled from a logarithmically growing culture over a 24 hour period using Hoechst 33342 enabled us to correlate DNA and chlorophyll content, and to observe changes in these values as a function of time. Previous studies have indicated that DNA, protein and chlorophyll contents fluctuate in these cultures in 24 hour cycles. Our goal is to analyze these parameters on a cell by cell basis using flow cytometry in order to obtain more detailed information about cell cycle events in this species. The blue fluorescence emissions of Hoechst 33342 do not overlap with the red emissions of chlorophyll (Yentsch *et al.* 1985, Davies and Kovacs 1990), and therefore make it an appealing choice for simultaneous analysis of DNA and chlorophyll content. For species with little or no autofluorescence in the green-orange range, fluorescein could in theory be added to allow three color analysis of DNA, protein and chlorophyll content.

Hoechst 33342 has been used to stain both viable and methanol fixed phytoplankton samples (Trask *et al.* 1982, Olson *et al.* 1983, Partensky *et al.* 1988). The fixative used in our procedures (1% paraformaldehyde, 1% glutaraldehyde in 0.5 M cacodylate buffer) is not comparable in effect to methanol fixation, and we observed some of the difficulties in using Hoechst that have been noted with viable samples: (i) DNA peaks with broad CV's, (ii) a slight drop in LFALS readings, indicating some morphologic affects, and (iii) an increase in LRFL readings for the dead cell and debris fraction. We were able to distinguish two separate DNA peaks despite the broad CV's and obtain valuable information without performing detailed cell cycle analysis. Further work to improve the staining of these fixed samples is needed. The increase in LRFL readings for cells with very dim fluorescence prior to staining may be due to secondary excitation of residual chlorophyll by Hoechst (Hamori *et al.* 1980, Premazzi *et al.* 1989). Previous sorting had shown these dimly red fluorescing cells to be non-viable. The fact that no changes in LRFL occurred for the viable cells indicates that energy transfer of this sort is not a significant problem in *C. meneghiniana* analysis. We will continue to explore and refine this technique in order to improve DNA peak resolution and determine optimal staining conditions.

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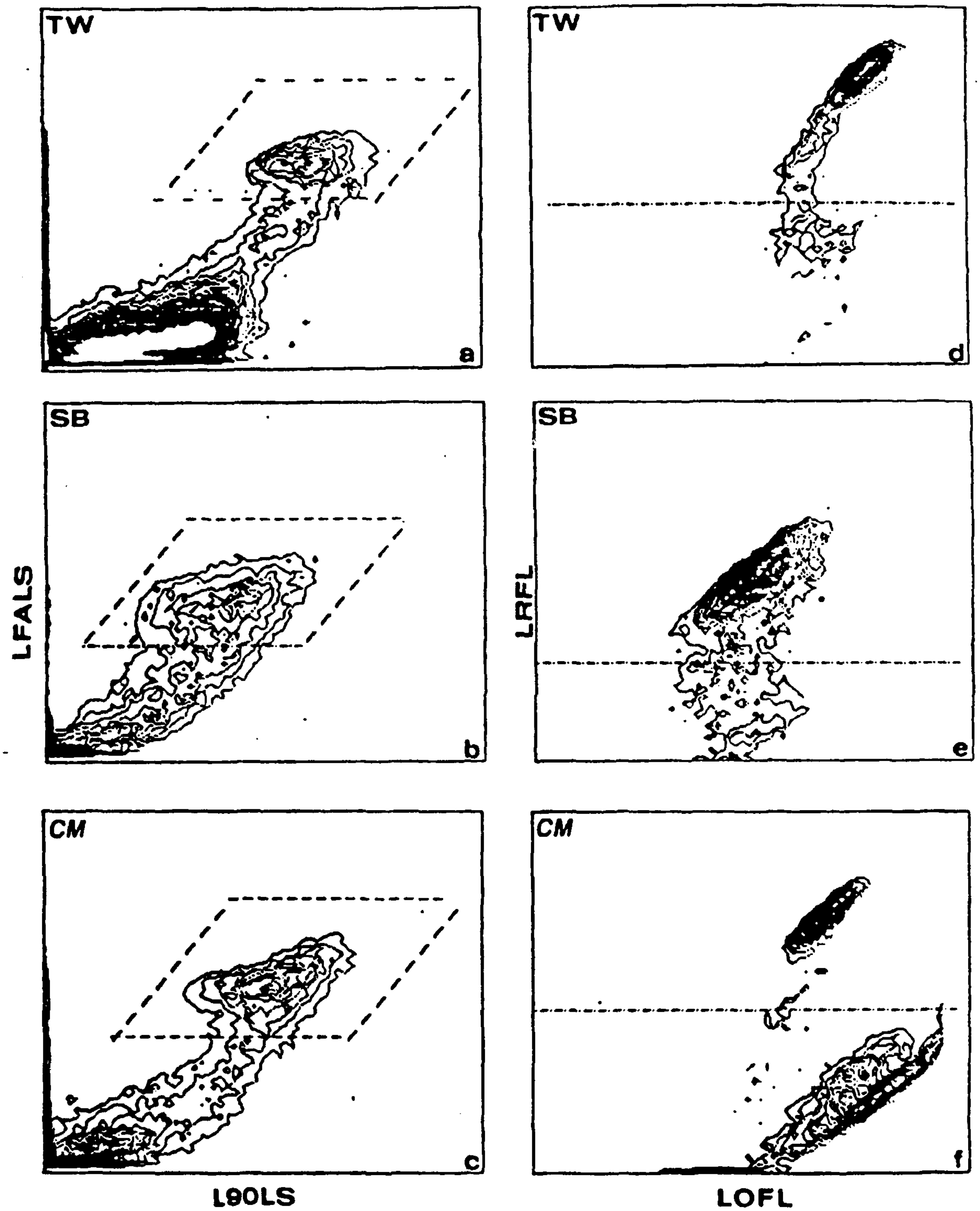


Figure 1. Light scatter and autofluorescence distributions for three diatoms on day 1 of fixation in 1% paraformaldehyde and 1% glutaraldehyde in .05 M cacodylate buffer. Cell types are: TW = *Thalassiosira weisflogii*, SB = *Stephanodiscus binderanus*, CM = *Cyclotella meneghiniana*. Figs. 1a,b,c = L90LS vs. LFALS for each culture; cells sorted from the boxed area were confirmed by microscope to be whole cells. Figs. 1d,e,f = LOFL vs. LRFL of this whole cell population. Cells sorted from above the line indicated on each distribution (high LRFL) were viable, while those below the line were non-viable.

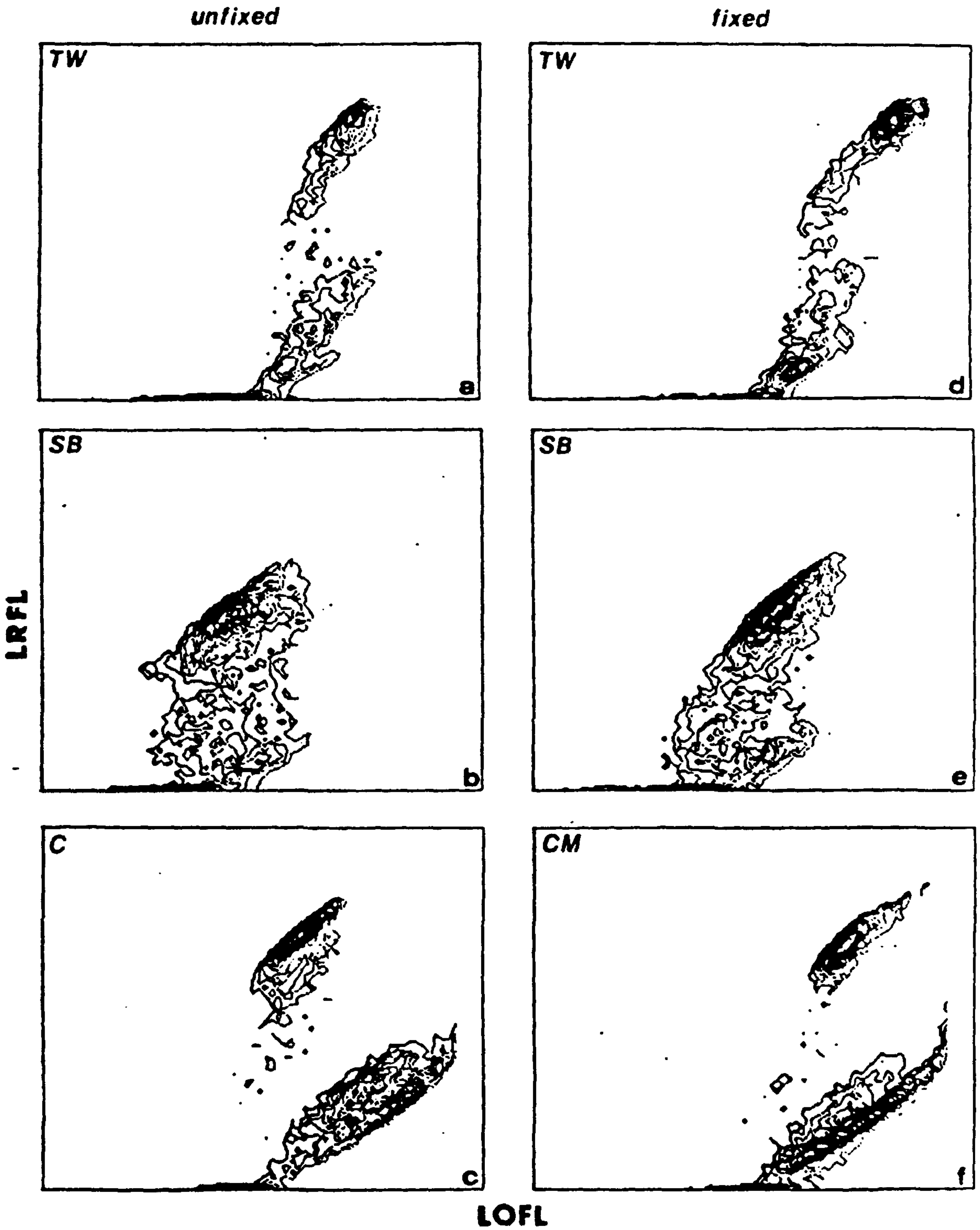


Figure 2. Comparison of autofluorescence distributions (LOFL vs. LRFL) for three diatoms after 28 days of storage at 4°C in the dark. Figs. 2a,b,c: unfixed cells. Figs. 2d,e,f: Cells fixed in 1% paraformaldehyde and 1% glutaraldehyde in .05 M cacodylate buffer.

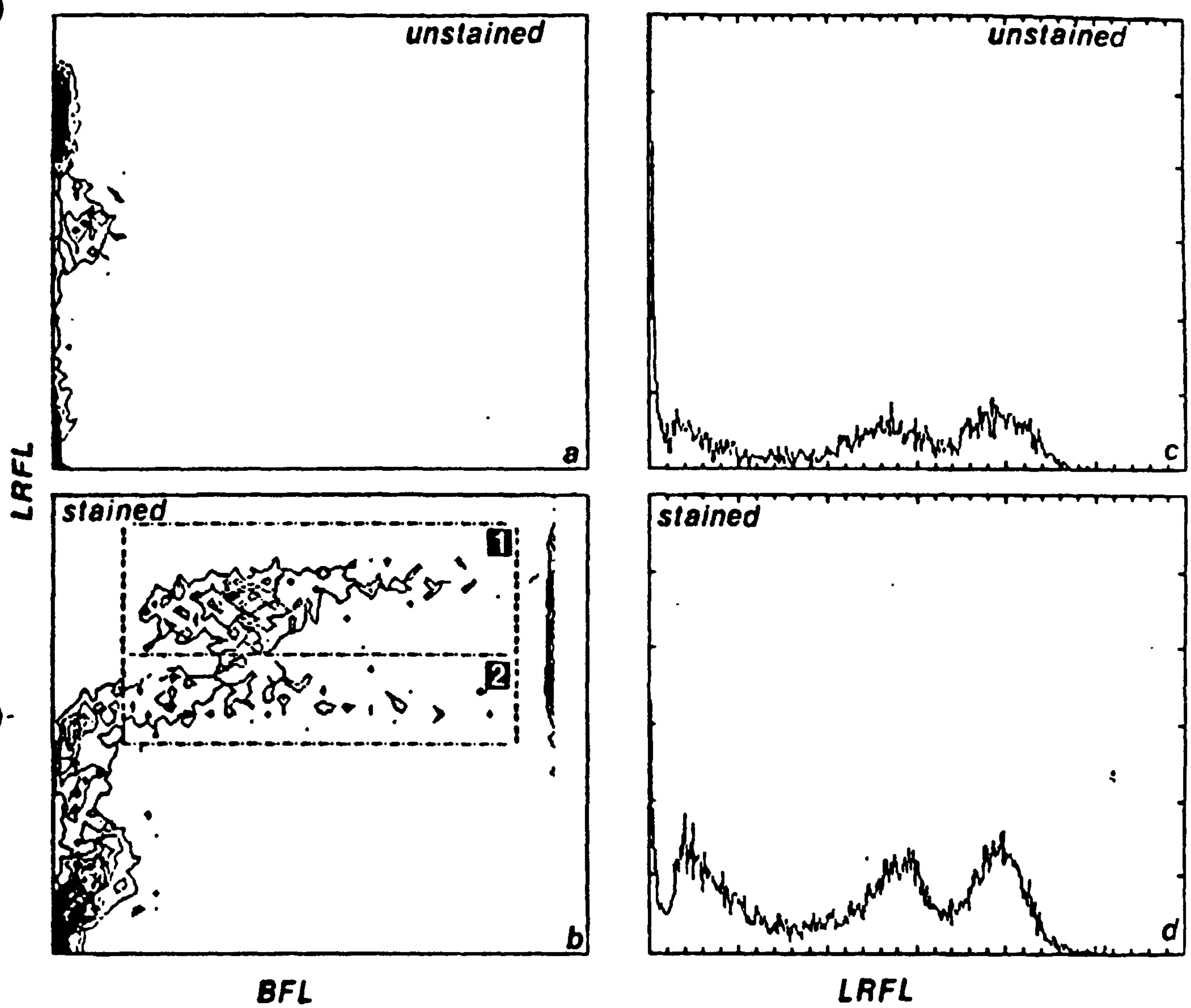


Figure 3. Comparison of unstained *C. meneghiniana* to cells from the same culture stained in 5 µg/mL Hoechst 33342 for 1 hour at room temperature. Figures a and b are 2P distributions of BFL (Hoechst 33342, DNA content) vs. LRFL (chlorophyll content) for unstained and stained cells, respectively. Figures c and d are 1P distributions of LRFL (chlorophyll content) resolved from the 2P distributions.

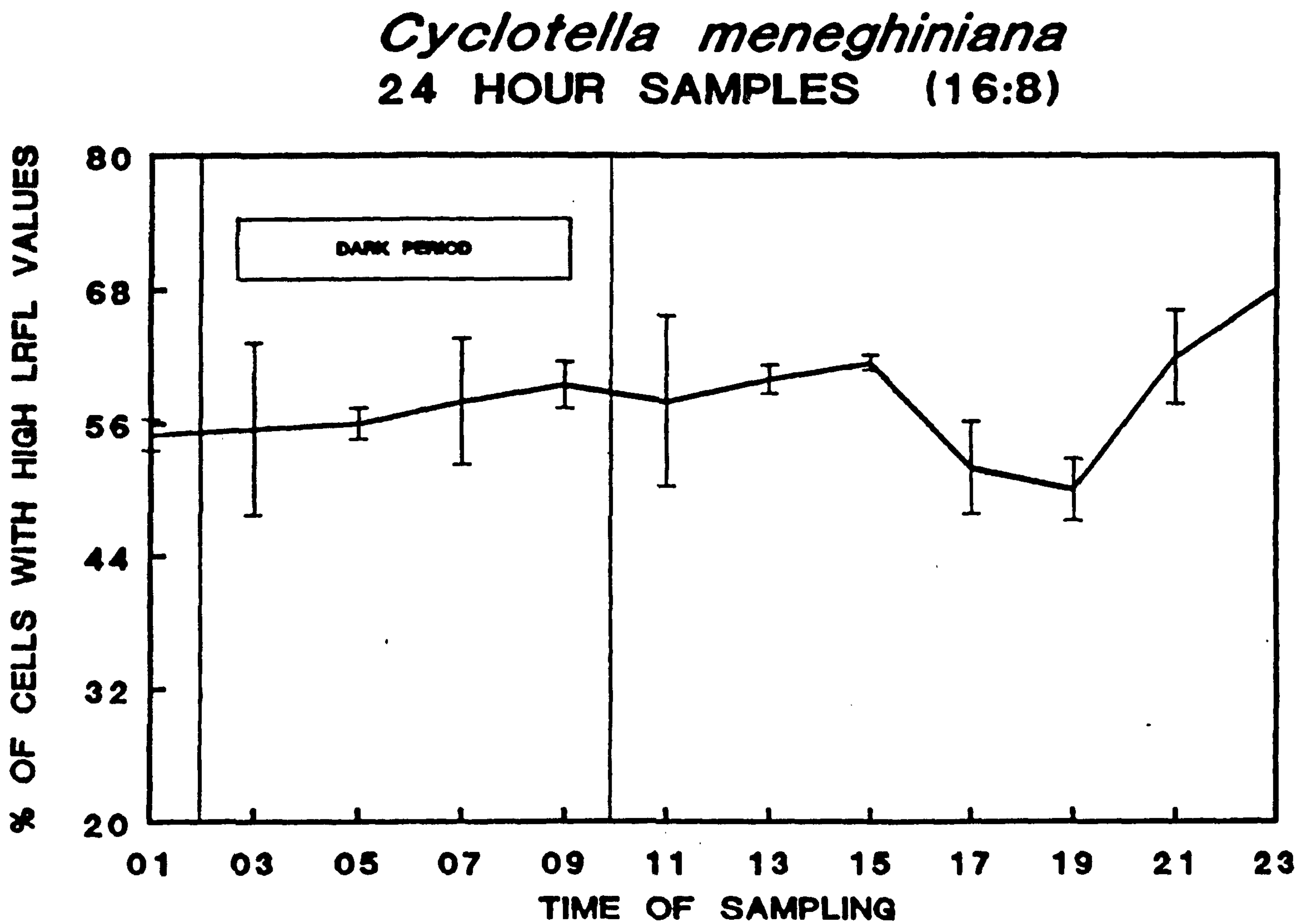


Figure 4. Percentage of cells with high red fluorescence and high DNA content versus time of day.

A2 –

# EFFECT OF GROWTH AND LIGHT/DARK CYCLES ON DIATOM LIPID CONTENT AND COMPOSITION

**Individual reprints for this chapter follow.**

*J. Phycol.* **24**, 1–7 (1988)

# EFFECT OF LIGHT CYCLE ON DIATOM FATTY ACID COMPOSITION AND QUANTITATIVE MORPHOLOGY[1]

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ABSTRACT

*Quantitative cytological and fatty acid composition was determined for the diatom* Cyclotella meneghiniana *Kütz. Data from four separate experiments were examined to elucidate changes that may occur with respect to daily photoperiod. Overall, fatty acid composition is similar to that reported for other diatoms with the exception that the C16 fatty acids constitute approximately 70% of all fatty acids. The major fatty acids are C14:0, 16:1, 16:0, 18:0, and 20:5. Fatty acids that are present in minor amounts are iso-14:0, iso-15:0, 15:0, 17:0, 18:4, 18:2, 18:1, 19:0, 20:0, 22:0, and 23:0.*

*Cytological composition is similar to that previously reported with the chloroplast and vacuole being the largest compartments within the cell. Changes in both cytological and fatty acid composition were studied with respect to the light/dark cycle. Chloroplast and lipid relative volume are greatest during the early part of the dark period. Nuclear relative volume is lowest in the dark and increases throughout the light period.*

*Total unsaturated fatty acids, including the C20:5 fatty acid, are lowest in the early part of the light period and highest in the dark. The sum of the C16 fatty acids remains constant at 70% of total fatty acids in the cells throughout the light/dark cycle, although percent composition of these two fatty acids shifts.*

*The data suggest that cyclical changes occur in both quantitative morphology and fatty acid composition with respect to daily photoperiod. The cells, although not rigidly synchronized, most likely divide in the latter part of the dark period or in the first hours of the light period. Lipids increase dramatically in the dark. The ecological implications of lipid storage are discussed in relation to lipophilic toxicants.*

*Key index words:* Cyclotella; *diatoms; fatty acids; light cycle; ultrastructure*

In phytoplankton, rapidly growing nutrient-sufficient cells incorporate most cell carbon into protein. However, under a variety of stress conditions, particularly those produced by nutrient limitation, more cell carbon is incorporated into lipid and carbohydrate storage products (Fogg 1956, Coombs et al. 1967b, Darley 1977, Shifrin and Chisholm 1981, Varum and Mykelstad 1984, Ben-Amotz et al. 1985, Smith and Geider 1985, Millie 1986, Ganf et al. 1986). Diatoms accumulate lipids or have altered fatty acid percent compositions as a function of culture age, silicon or nitrogen deficiency, or exposure to heavy metals (Ackman et al. 1964, Werner 1966, Coombs et al. 1967a, Opute 1974, Fisher and Schwarzenbach 1978, Shifrin and Chisholm 1981, Sicko-Goad et al. 1986a, b).

Accumulation of highly hydrophobic compounds appears to be related to lipid content. For example, Canton et al. (1977) found that for hexachlorocyclohexane, *Chlamydomonas* and *Dunaliella* had partition coefficients of 2700 and 1500 respectively, on a dry weight basis. However, when these values were normalized to lipid content, the partition coefficients obtained were 12,000 and 13,000. Thus it seems evident that lipid quantities and composition may change as a result of environmental and cultural perturbations and that lipids may be important in terms of uptake, storage, and toxicity of lipophilic compounds (Clayton et al. 1977, Boyles 1980, Hutchinson et al. 1980).

During a study of the effects of chlorinated benzenes on diatom ultrastructure and fatty acid composition, variability in both of these cellular parameters was noted that appeared to be related to the light/dark cycle. Since light is known to affect chloroplast structure (Brown and Richardson 1968, Meier and Lichtenthaler 1981) and fatty acid composition (Pohl and Zurheide 1979, Shifrin and Chisholm 1981), data from control cultures of four separate chlorinated benzene exposure experiments were pooled and compared to determine normal variability in these parameters with reference to the daily photoperiod. Results of this comparison are presented here.

## MATERIALS AND METHODS

The control data compiled in this paper were taken from four separate experiments in which the diatom *Cyclotella meneghiniana* Kütz. (Clone CYOH2) was exposed to chlorinated benzenes. During the exposure experiments, control cultures were manipulated exactly as experimental cultures. The four exposure experiments were conducted over a period of 12 months from October 1984 to September 1985.

Cells were grown in WC medium (Guillard 1975) on a 16:8 h LD cycle to logarithmic phase at 20° C with a light irradiance of 200 $\mu E \cdot m^{-2} \cdot s^{-1}$ as previously described (Sicko-Goad and Lazinsky 1986). Since experiments originally were designed to assess effects of chlorinated benzenes on ultrastructure and fatty acid composition, logarithmic phase cells were harvested at the following times after exposure to the toxicants: 10 min, 1 h, 2 h, 8 h, 24 h, and 5 days. These sampling times also included control cultures with no added toxicant, and these data were pooled to

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form the data base for the present comparison. Since the start of individual experiments did not coincide in all cases, data were divided into three time periods with respect to the daily LD cycle. Those samples described as "early light" were harvested during the first 8 h of light exposure; those described as "late light" were harvested during the second 8 h of light exposure; and those harvested during the 8 h dark period are referred to as "dark" samples. However, since these control data were pooled from toxicant exposure experiments and these experiments were not designed to assess cyclical changes with photoperiod, dark samples were obtained during the first three hours of the dark period, and most light samples were obtained during the second eight hours of the light period. In all cases, however, samples for electron microscopy and fatty acid analysis were withdrawn simultaneously.

*Electron microscopy.* Samples for electron microscopy were withdrawn into 50 mL sterile polypropylene centrifuge tubes. Fixative and buffer were added to give final concentrations of 1% paraformaldehyde, 1% glutaraldehyde, and 0.05 M cacodylate buffer at pH 7.2 as previously described (Sicko-Goad and Lazinsky 1986). Samples were post-fixed in 1% $OsO_4$, dehydrated in a graded ethanol-propylene oxide dehydration series and embedded in Embed 812. Sections were cut with a diamond knife, collected on formvar coated 200 mesh copper grids, and stained with aqueous uranyl acetate (Watson 1958). Sections were examined and photographed with a JEOL JEM 100B electron microscope operating at 80 kV.

The morphometric sampling scheme used has been described in detail (Sicko-Goad et al. 1977, 1984, 1986a) with precautions taken for unbiased sampling of unicells. The only exception to the previously described sampling scheme was that 25 photographs per individual time period in each of four experiments were examined. The point count per individual group of 25 cells was ca. 5000, and standard errors were within 10% of the mean. The number of individual control cultures analyzed by electron microscopy was 18, for a total cell count of 450. The distribution of the number of cells examined with respect to the three time periods defined is indicated in Table 3. All individual photographs within each time period were considered as independent estimates of the quantitative ultrastructure and were treated as individual replicates for that particular portion of the light/dark cycle.

*Fatty acid analysis.* Culture medium (125 mL) containing cells was withdrawn and filtered through GFC filters. The filter retaining the cells was placed in an aluminum foil wrapped vial containing 3.5 mL methanol/water (7:3) and 5% KOH solution. The solution was bubbled with nitrogen and boiled in a 60° C water bath for 2 h to complete deactivation and saponification. The fatty acids were extracted by filtering the above extract and removing other organics with 2 mL pentane. The solution was acidified with 2 mL 2.3 N HCl and the fatty acids were extracted 4 times with 1 mL pentane. Pooled fractions were then washed with 2 mL saturated NaCl and dried over anhydrous sodium sulfate (Schwarzenbach and Fisher 1978).

To esterify the fatty acids to methyl esters, the solvent was evaporated by nitrogen and the extract redissolved with 5 mL benzene in a vial with a Teflon-lined cap. Boron trifluoride (0.5 mL) was added and the solution was boiled for 4 min in a 60° C water bath. The solution was then washed with 10 mL distilled water and the fatty acid methyl esters (FAMEs) were extracted with hexane and dried over anhydrous sodium sulfate (Metcalfe and Schmitz 1961). The pooled extract was concentrated by a nitrogen stream to approximately 10 $\mu$L.

For quantification of the FAMEs, samples were analyzed on a Varian 3700 GC with a DB 5, (narrow bore, 1 $\mu$m film), 30 m fused silica column, equipped with a FID detector and a HP 3900A integrator. The GC/FID program consisted of the following conditions: Split mode (58:1), Injector temperature = 250° C, Detector temperature = 290° C. The column temperature program was hold 4 min at 225° C, 4° C/min rate, and 280° C hold 20 min. Separation and relative retention times were established with a standard FAME solution consisting of 12:0, 13:0, 14:0, 15:0, 16:1, 16:0, 17:0, 18:3, 18:2, 18:1, 18:0, 19:0, 20:3, 20:2, 20:1, 20:0, 21:0, 22:1, 22:0, 23:0, 24:1, and 24:0 fatty acids.

To verify fatty acids by gas chromatography/mass spectrometry (GC/MS), one of the triplicate samples from all time and experimental controls was pooled and concentrated. The concentrated sample (pooled from 24 individual replicates) and the standard FAME solution were analyzed on a Finnigan 4000 GC/MS system with an Incos data acquisition system and the same DB 5 column used in the GC/FID. The split mode was 5.4:1, with essentially the same temperature program as described above. The FAMEs were identified by use of NBS library, with the exception of the 18:4 and 20:5 fatty acids which were not present in the library. These FAMEs were identified by visual examination, and interpretation of the mass spectrum was based on the fragmentation pattern of the FAMEs.

To determine fatty acid percent composition, duplicate samples from 24 individual sampling times were examined. The distribution of the number of individual replicates within the established time periods discussed in this paper are indicated in Tables 1 and 2.

*Cell volume estimates.* Cell volume estimates were determined by light microscopic measurements of epoxy mounts of fixed cells. Measurements of length and diameter were made of 25 cells for each treatment. Consequently, n = 100 for early light period cells, n = 300 for late light period samples and n = 50 for dark period cells. Volumes were determined by assumption of a regular geometric shape by the relationship $V = \pi d^2 h/4$.

## RESULTS

*Fatty acids.* Typical fatty acid percent composition for all control cells is presented in Table 1. For *Cyclotella meneghiniana*, C16 fatty acids predominate, accounting for approximately 70% of all fatty acids in the cells. Although even numbered carbon fatty acids predominate, we also found four odd numbered carbon saturated fatty acids (15:0, 17:0, 19:0, and 23:0), with the C15 fatty acid predominant in the odd numbered chain lengths. The odd numbered carbon fatty acids constitute only approximately 2–3% of all fatty acids.

Changes were found in fatty acid composition when compared on the basis of time of sampling in the light/dark cycle (Table 2). Total unsaturated fatty acids were lowest in the early part of the light period and highest when sampled in the dark (Fig. 1). Conversely, saturated fatty acids predominated in the early light period. The sum of the C16 fatty acids remained constant throughout the entire LD cycle (ca. 70%).

*Quantitative ultrastructure.* The typical morphometric portrait of *C. meneghiniana* is presented in Table 3. The sum of vacuole, lipid, and polyphosphate relative volume is approximately 27%, which can be assumed to be the total vacuolar volume since lipid and polyphosphates are located in the vacuole. Likewise, chloroplast volume is the sum of chloroplast $V_V$ (relative volume) and chloroplast lipid $V_V$, and averages ca. 23.5%. Autophagic-like vacuoles are rare in control cells, comprising less than 0.5% of the total cell volume. The ranges of all other

TABLE 1. *Fatty acid identification and percent composition with standard errors of all* Cyclotella meneghiniana *control cells.*[a]

| Fatty acid | Structure | M.W. | % Composition |
|---|---|---|---|
| 14:0 | $C_{15}H_{30}O_2$ | 242 | 11.20 (0.25) |
| ISO13:0 | $C_{15}H_{30}O_2$ | 242 | 0.06 (0.02) |
| ISO14:0 | $C_{16}H_{32}O_2$ | 256 | 0.32 (0.04) |
| 15:0 | $C_{16}H_{32}O_2$ | 256 | 1.61 (0.08) |
| 16:1 (Δ9) | $C_{17}H_{32}O_2$ | 268 | 47.23 (1.30) |
| 16:0 | $C_{17}H_{34}O_2$ | 270 | 25.10 (1.00) |
| 17:0 | $C_{18}H_{36}O_2$ | 284 | 0.13 (0.04) |
| 18:4 (Δ6, 9, 12, 15) | $C_{19}H_{30}O_2$ | 290 | 0.75 (0.06) |
| 18:2 (Δ9, 12) | $C_{19}H_{34}O_2$ | 294 | 0.42 (0.09) |
| 18:1 (Δ9) | $C_{19}H_{36}O_2$ | 296 | 1.34 (0.15) |
| 18:0 | $C_{19}H_{38}O_2$ | 298 | 4.90 (0.53) |
| 19:0 | $C_{20}H_{40}O_2$ | 312 | 0.19 (0.19) |
| 20:5 (Δ5, 8, 11, 14, 17) | $C_{21}H_{32}O_2$ | 316 | 6.25 (0.34) |
| 20:0 | $C_{21}H_{42}O_2$ | 326 | 0.07 (0.06) |
| 22:0 | $C_{23}H_{46}O_2$ | 354 | 0.04 (0.04) |
| 23:0 | $C_{24}H_{48}O_2$ | 368 | 0.37 (0.10) |

[a] Overall composition was determined by averaging 48 replicates which were taken from four different experiments. Samples were averaged without respect to any time period.

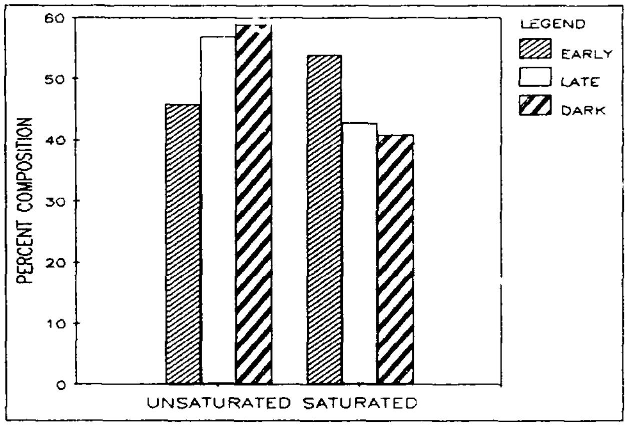



FIG. 1. Change in percent composition of total saturated and unsaturated fatty acids with respect to sampling period in the light/dark cycle.

categories are similar to earlier reported values (Sicko-Goad and Lazinsky 1986).

As with the fatty acids, definite changes in ultrastructure can be correlated with the LD cycle (Table 3). The chloroplast volume (chloroplast $V_V$ and chloroplast lipid $V_V$) increases throughout the light period and reaches its maximum in the dark. This increase is not due to changes in the chloroplast lipid since this decreases in the late part of the light period and in the dark (Fig. 6).

Lipid volume increases dramatically during the latter part of the light period and in the dark (Fig. 7). Again, as in the summary of all control cells (Table 3), total vacuole volume (vacuole $V_V$, lipid $V_V$, and polyphosphate $V_V$) remains constant at approximately 27–28%. Consequently, apparent changes are a direct result of changes in lipid volume. Nucleus relative volume is lowest in the dark and increases with time during the light period. All other cellular volume categories remain relatively constant throughout the LD cycle.

Micrographs of both dark and light period cells (Figs. 2–5) reveal that although individual sections may be found that exhibit some of the quantitative properties presented in Table 3, no single photograph can readily represent the population analyzed.

## DISCUSSION

Data presented here suggest that a moderate degree of division synchrony exists in control cultures of *Cyclotella meneghiniana*. This is especially significant when one considers that the data were pooled from four different experiments conducted over a period of 12 months under similar environmental conditions of light intensity and quality, photoperiod, temperature, and nutrient conditions. Although a moderate degree of division synchrony has been associated with silicon-starvation (Lewin et al. 1966, Coombs et al. 1967b) and by short periods of high light intensity (Chisholm et al. 1980, Chisholm and Costello 1980), the cell cycle in diatoms was not believed to be entrained by or phased to the LD cycle as it is in most other phytoplankton taxonomic groups (Chisholm et al. 1980).

Cytological observations that lead us to believe that on the whole, *Cyclotella* divides during the latter half of the dark period are the following: (1) cells are physically largest during the dark (the dark samples in these experiments were taken during the first three hours of the 8 h dark period), (2) nucleus, chloroplasts, and mitochondria increase in volume during the latter part of the light period, and (3) the numbers of chloroplasts and mitochondria also

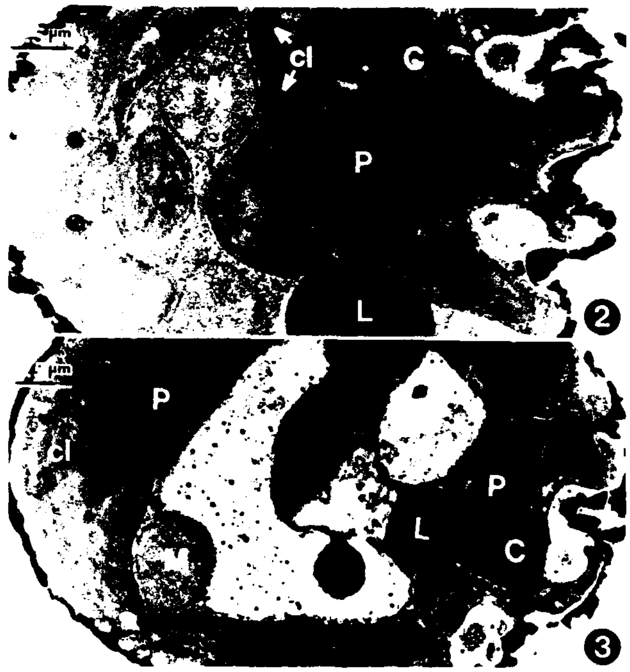
TABLE 2. *Variation in fatty acid percent composition with respect to light/dark cycle. Numbers in parentheses are standard errors.*

| Fatty acid | Early light period | Late light period | Dark period |
|---|---|---|---|
| 14:0 | 11.16 (0.40) | 11.29 (0.32) | 10.52 (0.76) |
| 15:0 | 1.60 (0.05) | 1.58 (0.10) | 1.80 (0.42) |
| 16:1 | 38.51 (2.34) | 49.12 (1.44) | 47.73 (3.91) |
| 16:0 | 32.09 (1.69) | 23.86 (1.11) | 22.75 (2.31) |
| 18:4 | 0.43 (0.09) | 0.80 (0.07) | 1.01 (0.10) |
| 18:1 | 1.56 (0.34) | 1.34 (0.18) | 0.93 (0.54) |
| 18:0 | 6.16 (1.14) | 4.62 (0.62) | 4.93 (2.36) |
| 20:5 | 5.05 (0.45) | 6.17 (0.40) | 9.34 (0.46) |
| All others | 3.45 (1.19) | 1.23 (0.20) | 1.00 (0.63) |
| Sample size | n = 8 | n = 36 | n = 4 |

TABLE 3. *Morphometric summary of all control data and changes in values with respect to light/dark cycle. Numbers reported are* $N_V$ = *number per volume*[a]; $V_V$ = *relative volume.*[b] *Numbers in parentheses are standard errors.*

| | Early | Late | Dark | All |
|---|---|---|---|---|
| Chloroplast $V_V$ | 21.49 (0.69) | 22.10 (0.65) | 28.15 (1.62) | 22.64 (0.50) |
| Chloroplast lipid $V_V$ | 1.09 (0.09) | 0.78 (0.05) | 0.81 (0.10) | 0.86 (0.04) |
| Mitochondria $V_V$ | 3.54 (0.23) | 3.94 (0.18) | 3.50 (0.31) | 3.80 (0.13) |
| Av-like $V_V$[c] | 0.57 (0.20) | 0.34 (0.06) | 0.17 (0.07) | 0.37 (0.06) |
| Lipid $V_V$ | 3.05 (0.45) | 5.96 (0.45) | 9.97 (2.01) | 5.76 (0.40) |
| Vacuole $V_V$ | 23.05 (1.13) | 19.05 (0.56) | 17.13 (1.71) | 19.73 (0.50) |
| Other $V_V$ | 17.62 (0.58) | 17.37 (0.37) | 15.56 (0.83) | 17.22 (0.29) |
| Nucleus $V_V$ | 14.87 (0.89) | 16.56 (0.66) | 10.64 (1.63) | 15.53 (0.52) |
| Frustule $V_V$ | 12.06 (0.30) | 11.55 (0.23) | 11.94 (0.41) | 11.71 (0.17) |
| Polyphosphate $V_V$ | 1.67 (0.24) | 1.45 (0.12) | 1.63 (0.31) | 1.52 (0.10) |
| Fibrous Vacuole $V_V$ | 0.98 (0.12) | 0.90 (0.14) | 0.50 (0.25) | 0.87 (0.10) |
| Chloroplast $N_V$ | 0.131 (0.007) | 0.162 (0.008) | 0.137 (0.011) | 0.152 (0.006) |
| Chloroplast lipid $N_V$ | 18.14 (2.11) | 27.37 (8.22) | 15.64 (2.71) | 24.02 (5.51) |
| Mitochondria $N_V$ | 0.071 (0.005) | 0.101 (0.006) | 0.096 (0.011) | 0.094 (0.004) |
| Polyphosphate $N_V$ | 0.883 (0.139) | 0.587 (0.070) | 0.680 (0.153) | 0.654 (0.058) |
| Sample size | $n$ = 100 | $n$ = 300 | $n$ = 50 | $n$ = 450 |
| Average cell volume | 253 ± 6 $\mu m^3$ | 277 ± 4 $\mu m^3$ | 297 ± 10 $\mu m^3$ | 274 ± 3 $\mu m^3$ |

[a] $N_V$ = no. per $\mu m^3$.
[b] $V_V$ = relative volume is comparable to percent composition and is expressed as a percentage of the total cell volume. Absolute volume can be determined by multiplying relative volume times the average cell volume.
[c] Av = autophagic-like vacuole.



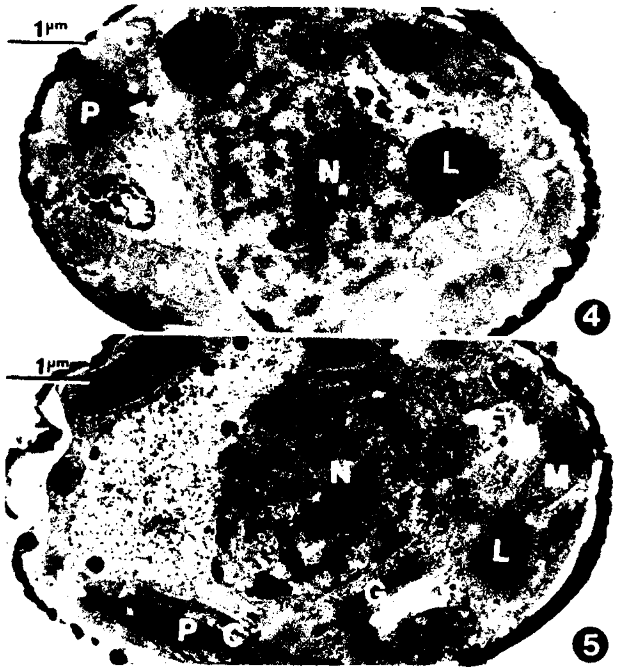

FIGS. 2, 3. Electron micrographs of cells sampled during the dark. Note large chloroplasts (C) with pyrenoid (P) and lipid droplets (cl). Mitochondria (M) and lipid (L) are also present.

increase during the late light period and are at their lowest numbers during the early light period. Gaffal et al. (1982) found that absolute nuclear volume increases during the period of interphase growth up to the initial stages of mitosis.

While these data suggest that the population of cells, on the whole, divides at night, it also demonstrates that the cells are not rigidly synchronized to the light/dark cycle, in agreement with Chisholm and Costello (1980). Non-synchronous cell cycle timing can be advantageous where diatom populations are subjected to widely varying environmental parameters and can be a survival strategy (Chisholm 1981, Cosper 1982, Sicko-Goad 1986, Sicko-Goad et al. 1986b).

Data presented here are in agreement with those presented by Atkinson et al. (1974) for synchronized cultures of *Chlorella fusca* var. *vacuolata*. For example, Atkinson et al. (1974) reported that starch reserves are depleted during the process of cytokinesis which occurs during the dark. They also reported that the first starch to disappear was that around the pyrenoid and that consumption began during the last few hours of photosynthesis. Puiseux-Dao (1981) reported an increase in chloroplast storage droplets (both numbers and size) at the beginning of the light period. Similarly, in *Cyclotella* both vacuolar and chloroplast lipid reserves are highest during the early part of the dark period and lowest in the early light period. Thus it appears that storage products accumulate in the light and are utilized in the dark period to sustain cytokinesis and respiration in the absence of photosynthesis. The first storage products to be utilized in *Cyclotella* are those in the chloroplast although the amount of storage material in the chloroplast is insignificant on a volume basis.

The increase in chloroplast volume during the late light period and early in the dark period is most probably a result of two processes occurring simultaneously: (1) organelle and/or cell division and (2) light reduction. Growth of chloroplasts during the cell cycle was documented by Atkinson et al. (1974) who found the largest relative volumes (an increase of ca. 20%) of chloroplast prior to division. However, the cell division process may not entirely account for the increase in chloroplast volume (ca. 30%) that occurs in *Cyclotella* in the dark, assuming that division most often occurs during the latter part




FIGS. 4, 5. Electron micrographs of cells sampled during the early light period. FIG. 4. Section through the central portion of the cell showing the chloroplast (C) with pyrenoid (P) and lipid droplets (cl). Nucleus (N), mitochondria (M), and Golgi (G) vesicles. FIG. 5. Grazing section through the vacuolar region showing the distribution of small polyphosphate bodies (pp) in the vacuole and fibrous vacuoles (FV) in the cytoplasm adjacent to the Golgi.

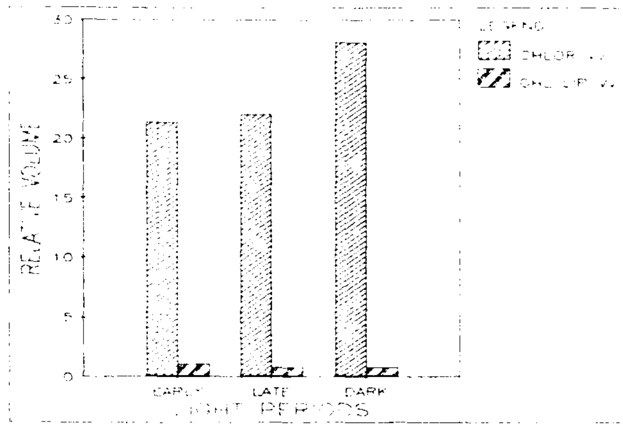



FIG. 6. Change in relative volume (percent of total cell volume) in chloroplast and chloroplast lipid with respect to the light period sampled.

of the dark period. Brown and Richardson (1968) found that in a variety of algae, volumes of both cells and chloroplasts decreased with increasing light intensity and in most cases, pigment content varied directly with chloroplast size. Messer and Ben-Shaul (1972) demonstrated that in *Peridinium*, cell size and chlorophyll content increased during early growth and decreased with culture age. Aged chloroplasts were narrower and had fewer thylakoids. Holmes (1966) demonstrated that, in four marine diatoms, nutrient deficiencies resulted in both a cessation of chlorophyll *a* synthesis and a concomitant decrease in the number of chloroplasts resulting from chloroplast division failure rather than degeneration.

Chloroplasts exposed to light are characterized by more dense thylakoid stacking and higher chlorophyll contents (Tageeva et al. 1971, Puiseux-Dao 1981). Since pigment synthesis is largely confined to the light period (Jörgensen 1966, Eppley and Coatsworth 1966, Eppley et al. 1967, Cosper 1982), it seems reasonable to assume that during the dark period pigment content is lower, resulting in less efficient thylakoid packing and an overall increase in chloroplast volume. However, the increase in chloroplast volume that is evident during the latter part of the light cycle is probably a result of enlargement before division.

The fatty acid composition of *Cyclotella meneghiniana* is similar to reports of fatty acid composition for other diatoms (Kates and Volcani 1966, DeMort et al. 1972, Orcutt and Patterson 1975, Fisher and Schwarzenbach 1978, Ben-Amotz et al. 1985, Nichols et al. 1986, Smith et al. 1986). In addition to the major fatty acids, we found two branched fatty acids (iso-13:0 and iso-14:0) consistently present but in very low quantities. DeMort et al. (1972) reported the presence of a branched C14 fatty acid in *Chlamydomonas* and a branched C15 fatty acid in seven different algae, including three diatoms. The presence of palmitoleic and palmitic acids as dominants is not suprising. However, in *Cyclotella* these two fatty acids consistently comprise approximately 70% of all fatty acids.

Fisher and Schwarzenbach (1978) demonstrated that C16:0 and C16:1 fatty acids in *Thalassiosira pseudonana* were stored in triglycerides and were partially or completely oxidized when cells were in the dark for prolonged periods of time and needed energy for metabolism. Similarly, Otsuka and Morimura (1966) found that *Chlorella ellipsoidea* oxidized large amounts of non-polar fatty acids when the cells

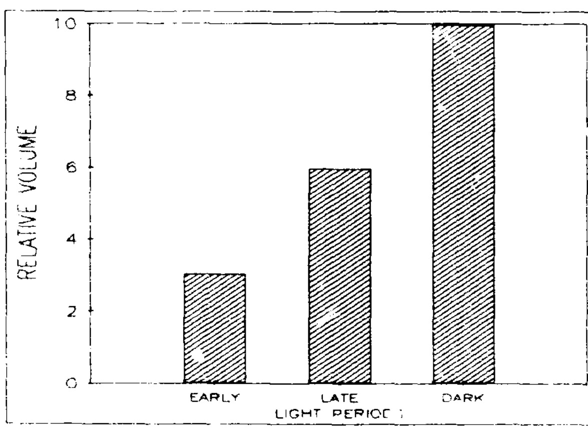



FIG. 7. Change in relative volume (percent of total cell volume) of lipid with respect to the light period sampled.

needed energy (i.e. in the dark or during cell division). It would appear from our data that the increase in lipid volume as darkness approaches parallels the increase in C16:1 and C20:5 fatty acids, and that the levels of these two fatty acids decline in the early light period.

Previous reports of alterations in fatty acid composition with respect to culture conditions agree remarkably with our data. For example, Kates and Volcani (1966) suggested that the requirement for photosynthesis in diatoms is for a certain degree of unsaturation, which is possibly supplied by the C20:5 fatty acid. This fatty acid in *Cyclotella* increases late in the light period, reaches its maximum in the dark, and is at its lowest levels in the early part of the light period. In *Cyclotella*, as in many diatoms, (DeMort et al. 1972), linolenic acid is absent. However, all unsaturated fatty acids are lowest early in the light cycle, presumably when photosynthesis is at its maximum rate, and highest in the dark.

Under stress conditions such as nutrient limitation or high light intensity, algae accumulate lipids (Shaw 1966, DeMort et al. 1972, Shifrin and Chisholm 1981). Similarly, rejuvenating resting cells of the diatom *Melosira granulata* produce large quantities of lipids during the active photosynthetic stage and utilize these storage products prior to cell division (Sicko-Goad 1986, Sicko-Goad et al. 1986b). Schlenk et al. (1960) and Milner (1948) found that as lipid content increased in *Chlorella* due to nutrient stresses, the degree of unsaturation of fatty acids decreased significantly. This trend is not apparent in the organism we studied, which was nutrient sufficient. Shifrin and Chisholm (1981) demonstrated that changes in total lipids over a daily cycle are associated directly with cell growth and reproduction. It is most probable that polyunsaturated acids are replenished in the dark for resumption of photosynthesis upon exposure to light. Studies by Pohl and Zurheide (1979) and Tornabene (1981) indicate that in *Chlorella* and *Euglena* an increase in total lipids corresponds with formation of polyunsaturated fatty acids.

The data presented here indicate that cyclical changes occur in both quantitative morphology and fatty acid composition with respect to the daily photoperiod. The most dramatic changes include increases in chloroplast and lipid volumes and increases in the C16:1 and C20:5 fatty acids in the dark. Because the changes are so dramatic, it seems apparent that precautions should be taken to separate cell-cycle linked changes from changes that result from stress factors such as environmental perturbations. Studies related to cell cycle changes may aid in elucidating metabolic changes and/or accommodations that occur as a result of toxicant exposure. For example, lipophilic compounds may be stored more when phytoplankton are in the dark or nutrient stressed. Consequently, release of the toxicant would be linked to resumption of active growth or cell division. We have shown synergistic relationships between polyphosphate and heavy metal accumulation (Sicko-Goad and Lazinsky 1986) where metals are sequestered in polyphosphate and are made available to the metabolic pool only upon phosphate limitation or starvation. Similarly, environmental conditions may produce physiological responses that make phytoplankton more susceptible to partitioning of lipophilic compounds. In both cases, sequestering of toxicants in storage products may result in toxicant stress to phytoplankton when cells are spatially and temporally removed from the toxicant source and are growing under different environmental parameters. Understanding the normal metabolic processes of phytoplankton will lead to a better understanding of the mode of action of toxicants.

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# EFFECT OF LIPID COMPOSITION ON THE TOXICITY OF TRICHLOROBENZENE ISOMERS TO DIATOMS

## I. Short-term Effects of 1,3,5-trichlorobenzene

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**Abstract.** Cultures of *Cyclotella meneghiniana* and *Melosira varians* were split into four flasks. Two flasks of each taxon were used for exposure experiments and two were used as controls. Exposure to 1,3,5-trichlorobenzene was initiated in one flask of each taxon in the 11th hour of the light period on a 16:8 h L/D regime. Another exposure was initiated four hours later in the same day (the 15th hour of the light period) in the second experimental flask of each taxon. These experiments were conducted at 15°C and 20°C for 2-4 days and were analyzed for lipid class composition. *Cyclotella* demonstrated some short-term changes in lipid class composition when exposures were initiated in the 11th hour of the light cycle at 20°C and in both exposures at 15°C. In contrast, short-term effects were only observed in *Melosira* when the exposure was initiated in the 11th hour of the light period at 20°C. Control cells demonstrated periodicities in lipid composition that appeared to be entrained with the light/dark cycle. The cultures that demonstrated toxicity effects were characterized by low triacylglycerol content, low neutral/polar lipid ratios, and a pattern of chlorophyll synthesis at the time of exposure. It is suggested that rapidly growing cells are more susceptible to short term effects of this trichlorobenzene isomer. Furthermore, timing of the initial exposure may alter toxicity results and this is a result of lipid class composition at the time of exposure.

## INTRODUCTION

Studies on the diatom *Cyclotella meneghiniana* (Sicko-Goad *et al.* 1988, 1989a,b,c,d) demonstrated that lipid content and fatty acid composition varied with time of day when the algae were grown on a 16:8h light/dark cycle. Furthermore, it was observed that the timing of the exposure to chlorinated benzenes altered the expected results. Lipophilicity and chemical reactivity predicted on structure of the compounds had less effect than the time of day the exposure was initiated. We hypothesized that toxicity effects observed might be due to either (1) increased partitioning of the lipophilic toxicant due to increased levels of total extractable lipid at certain times of the day or (2) changes in lipid fractions, especially alterations in total polar or total neutral lipids resulted in increased effects. To test this hypothesis, we conducted two sets of exposure experiments in which diatom cultures were split into separate flasks and exposures to chlorinated benzenes were initiated at two different times of day and at two different temperatures.

## MATERIALS AND METHODS

### *Algal Cultures*

*Cyclotella meneghiniana* Kütz. clone CyOH2 was obtained from Dr. S. S. Kilham of the Department of Biological Sciences at the University of Michigan. *Melosira varians* C.A. Ag. was obtained from Dr. H. Vanderplug of the Great Lakes Environmental Research Lab, NOAA (Ann Arbor, Michigan). Cultures were maintained in WC medium (Guillard 1975) on a rotary shaker table in a growth chamber set at 20°C on a 16:8 hour light/dark cycle at 50 $\mu$ein/$m^2$/sec. For experimental purposes, exponentially growing cells were evenly subdivided among four 2-L extracted and acid washed glass flasks.

Two sets of exposure experiments were conducted. The first experiment was conducted in July 1989 and was an exposure of *Cyclotella meneghiniana* and *Melosira varians* to 0.245 ppm 1,3,5-trichlorobenzene at 20°C. For this experiment, the trichlorobenzene was made up as a stock solution and methanol was used as a carrier. Stock cultures of each species were evenly divided among four flasks and were diluted with culture medium to a density of approximately 100,000 cells/mL. Two of the flasks were used as manipulation controls. All samples were withdrawn by a dry air pumping apparatus to minimize loss due to volitization of toxicant. Controls were sampled similarly. Methanol was added to the control flasks at a concentration of 0.75%, the same concentration in exposure flasks. 1,3,5-trichlorobenzene was added to one exposure flask at 2 pm, which corresponded to the 11th hour of the light cycle. The isomer was added to the second exposure flask at 6 pm, which corresponded to the 15th hour of the light cycle.

The second exposure experiment was identical in all respects to the first experiment with the exception of temperature, which was 15°C. Stock cultures for this experiment were acclimated at 15°C for two months prior to the exposure.

Aliquots from thoroughly mixed 2-L flasks were withdrawn at times 0, 2, 24 and 48, and for the 15°C experiment, 96h. 100 mL were withdrawn per sample. All samples were taken in duplicate, filtered onto prewashed and preweighed Gelman A/E glass fiber filters, air dried, then dried in a vacuum oven at 60°C for 24 h. Filters were reweighed to determine dry weight and frozen for subsequent lipid extraction.

For lipid analysis, frozen filters were placed in pre-extracted thimbles and extracted with chloroform/methanol (2:1 v/v) for at least 12h in a micro-Soxhlet (Orcutt and Patterson 1975). The extract was concentrated, redissolved in chloroform and washed with water in a separatory funnel, dried under a nitrogen stream in a pre-weighed Teflon-lined screw cap amber vial and weighed for total gravimetric lipid. Samples were flushed with nitrogen and frozen for lipid class analyses.

Samples were unfrozen and redissolved in methylene chloride to concentrations of 20-50 μg lipid in spotting volumes of 10-20 μL for lipid class analysis. The extracts were spotted with Hamilton syringes onto cleaned and blank-scanned silica coated chromarods (type SIII). Rods were held in a frame and developed, then scanned in an Iatroscan Mark IV TLC-FID (RSS Inc., Costa Mesa, CA.) system using the development scheme of Parrish (1986). The development was three staged:

1. The rods were developed in 50 mL solution of hexane, diethyl ether, and formic acid (98:2:0.5) for 30 min, conditioned for 5 min, followed by redevelopment in the same solvent system for 25 min. Rods were then partially scanned for hydrocarbons, wax/sterol esters, and ketones. β-carotene migrates with hydrocarbons. Wax and sterol esters co-elute in this system.
2. Rods were then reconditioned and developed in 50 mL of a solution of hexane, diethyl ether and formic acid (80:20:0.1) for 50 min. Rods were scanned for triacylglycerols, free fatty acids, alcohols and sterols. Since triacylglycerols and free fatty acids are difficult to separate if the load on the chromarod is greater than 10 μg, the area of the split peak has been combined into one class labelled TG/FFA. Diacylglycerols have an Rf value near that of sterols. No diacylglycerol peaks were observed in any samples.
3. The third development consisted of two 15-min developments in 100% acetone, followed by two 10-min developments in dichloromethane, methanol and water (5:4:1). Rods were then scanned for chlorophyll *a*, acetone-mobile polar lipids, and a class containing phospholipids and acetone-immobile polar lipids. During the last scan, an FTID detector was also used for the additional detection of N in Chl *a* and phospholipids. The AMPL fraction may contain monoglycerides and glycolipids. Chlorophyll data from the TLC-FTID method were compared with standard fluorometric measurements from two experiments. The data were in agreement within one standard error of the TLC-FTID method.

Quantitative determinations of lipid class composition are based on dose-response calibration equations generated by analysis of a wide range of concentrations of standards for each lipid class (Parrish and Ackman 1985). Standards were obtained from Sigma at a purity of >99% for lipid class composition studies and are listed in Table 1. All lipid class composition determinations were run in duplicate. Thus, data reported are averages of four replicates for each time determination.

**Table 1.** Standards used for calibration of chromarods

| Lipid class | Abbrev. | Standards |
|---|---|---|
| Aliphatic hydrocarbons | HC | Nonadecane |
| Wax and sterol esters | WE/SE | Palmitic acid stearyl ester (Stearyl palmitate) |
| Ethyl ketone | KET | 3-Hexadecanone |
| Triacylglycerol | TG | Tripalmitin (Glyceryl tripalmitate) |
| Free fatty acids | FFA | Palmitic acid (hexadecanoic acid) |
| Free alcohol | ALC | Cetyl alcohol (hexadecanol) |
| Free sterol | ST | Cholesterol (5(6)-cholesten-3-ol) |
| Phospholipids | PL | L-α-phosphatidylcholine, diheptadecanoyl |
| Chlorophyll | Chl *a* | Chlorophyll *a* |

**Table 2.** Lipid class composition of *Cyclotella meneghiniana*. Control and exposure cultures. Growth conditions 16:8 h L/D at 20°. 2 pm samples were in the 11th hour of the light cycle; 6 pm samples were in the 15th hour of the light cycle. Sample times are annotated with actual time of day. Selected ratios and % composition presented as averages and standard error ( )

| Sample time | HC | | WE/SE | | TG/FFA | | ALC | | ST | | Chl *a* | | Ampl | | PL | | Chl *a*/Neut | | Neut/Polar | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| hr of day | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. |
| (A) = Exposure initiated at 2 pm | | | | | | | | | | | | | | | | | | | | |
| t = 0 | 5.7 | | 2.1 | | 66.9 | | 1.4 | | 1.3 | | 11.7 | | 5.1 | | 5.8 | | 0.18 | | 4.0 | |
| 2 pm | (2.2) | | (0.5) | | (4.3) | | (0.8) | | (0.6) | | (1.4) | | (1.0) | | (2.6) | | (0.01) | | (0.6) | |
| t = 2 | 0.7 | 2.2 | 0.0 | 1.8 | 83.7 | 75.5 | 0.0 | 0.0 | 1.2 | 1.5 | 8.1 | 10.3 | 5.4 | 6.6 | 0.9 | 2.1 | 0.09 | 0.14 | 6.0 | 6.0 |
| 4 pm | (0.0) | (0.8) | (0.0) | (0.9) | (0.0) | (7.7) | (0.0) | (0.0) | (0.0) | (0.5) | (0.0) | (3.8) | (0.0) | (1.6) | (0.0) | (0.4) | (0.00) | (0.06) | (0.0) | (2.0) |
| t = 24 | 0.2 | 2.2 | 0.7 | 1.2 | 79.4 | 78.5 | 0.0 | 0.0 | 2.5 | 1.3 | 12.8 | 7.3 | 2.2 | 3.7 | 2.2 | 5.7 | 0.16 | 0.10 | 6.7 | 5.0 |
| 2 pm | (0.1) | (0.7) | (0.3) | (0.3) | (5.1) | (8.0) | (0.0) | (0.0) | (0.6) | (0.8) | (3.5) | (2.3) | (0.6) | (1.5) | (0.6) | (2.7) | (0.05) | (0.03) | (2.7) | (3.0) |
| t = 48 | 7.8 | 5.9 | 5.5 | 5.4 | 59.7 | 59.5 | 0.6 | 0.7 | 2.6 | 3.3 | 8.6 | 12.6 | 4.4 | 5.5 | 10.6 | 7.1 | 0.12 | 0.17 | 3.4 | 3.0 |
| 2 pm | (1.6) | (1.0) | (0.9) | (0.8) | (5.8) | (2.6) | (0.4) | (0.4) | (0.5) | (0.8) | (1.8) | (3.2) | (1.0) | (2.5) | (1.1) | (2.8) | (0.03) | (0.04) | (0.5) | (0.3) |

(continued)

**Table 2.** (continued)

| Sample time | HC | | WE/SE | | TG/FFA | | ALC | | ST | | Chl *a* | | Ampl | | PL | | Chl *a*/Neut | | Neut/Polar | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| hr of day | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. |
| (B) = Exposure initiated at 6 pm | | | | | | | | | | | | | | | | | | | | |
| t = 0 | 2.3 | | 0.7 | | 68.7 | | 0.0 | | 2.9 | | 14.6 | | 8.8 | | 2.1 | | 0.2 | | 3.0 | |
| 6 pm | (0.4) | | (0.4) | | (1.8) | | (0.0) | | (0.7) | | (2.1) | | (0.8) | | (0.2) | | (0.04) | | (0.3) | |
| t = 2 | 1.3 | 2.5 | 3.6 | 4.1 | 69.3 | 67.4 | 0.1 | 0.3 | 1.0 | 2.3 | 17.7 | 15.4 | 5.4 | 6.1 | 1.6 | 2.0 | 0.24 | 0.21 | 3.2 | 3.7 |
| 8 pm | (0.1) | (0.6) | (0.1) | (1.4) | (2.6) | (5.8) | (0.1) | (0.3) | (0.6) | (0.4) | (2.6) | (3.3) | (1.0) | (1.4) | (0.4) | (0.6) | (0.04) | (0.05) | (0.5) | (0.9) |
| t = 24 | 1.9 | 3.8 | 2.1 | 5.4 | 70.6 | 57.4 | 0.0 | 0.4 | 3.9 | 2.8 | 13.9 | 15.5 | 4.0 | 5.4 | 3.5 | 9.3 | 0.18 | 0.23 | 3.7 | 2.5 |
| 6 pm | (0.5) | (0.7) | (0.9) | (0.4) | (1.6) | (3.5) | (0.0) | (0.3) | (0.5) | (0.8) | (1.3) | (3.6) | (1.3) | (1.1) | (0.8) | (0.6) | (0.02) | (0.03) | (0.2) | (0.4) |
| t = 48 | 4.9 | 3.1 | 6.1 | 10.0 | 50.8 | 53.9 | 1.4 | 0.8 | 4.7 | 4.3 | 9.7 | 13.0 | 3.6 | 5.9 | 18.8 | 9.0 | 0.15 | 0.19 | 2.2 | 2.7 |
| 6 pm | (0.7) | (0.6) | (0.8) | (2.5) | (5.5) | (1.9) | (0.3) | (0.5) | (0.8) | (1.5) | (1.8) | (3.2) | (1.8) | (2.8) | (3.1) | (1.3) | (0.03) | (0.05) | (0.3) | (0.4) |

**Table 3.** Lipid class composition of *Cyclotella meneghiniana*. Control and exposure cultures. Growth conditions 16:8 h L/D at 15°. 2 pm samples were in the 11th hour of the light cycle; 6 pm samples were in the 15th hour of the light cycle. Sample times are annotated with actual time of day. Selected ratios and % composition presented as averages and standard error ( )

| Sample time | HC | | WE/SE | | TG/FFA | | ALC | | ST | | Chl *a* | | Ampl | | PL | | Chl *a*/Neut | | Neut/Polar | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| hr of day | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. |
| (A) = Exposure initiated at 2 pm | | | | | | | | | | | | | | | | | | | | |
| t = 0 | 4.2 | | 37.1 | | 27.1 | | 4.3 | | 5.8 | | 5.7 | | 6.8 | | 8.9 | | 0.07 | | 3.9 | |
| 2 pm | (0.7) | | (4.2) | | (3.7) | | (0.7) | | (1.7) | | (0.9) | | (2.2) | | (0.6) | | (0.01) | | (0.4) | |
| t = 2 | 2.8 | 6.3 | 36.8 | 5.9 | 33.5 | 65.4 | 2.2 | 0.0 | 2.8 | 3.6 | 3.6 | 1.1 | 4.4 | 12.2 | 13.8 | 5.6 | 0.05 | 0.01 | 3.8 | 4.5 |
| 4 pm | (0.3) | (2.5) | (2.7) | (1.8) | (0.6) | (3.0) | (0.2) | (0.0) | (0.5) | (0.7) | (0.4) | (0.8) | (0.7) | (1.2) | (3.0) | (1.4) | (0.00) | (0.01) | (0.7) | (0.6) |
| t = 24 | 5.0 | 4.8 | 33.6 | 30.2 | 35.0 | 40.3 | 2.1 | 2.2 | 4.4 | 2.6 | 4.1 | 5.1 | 7.9 | 3.1 | 7.9 | 11.6 | 0.05 | 0.06 | 4.2 | 4.2 |
| 2 pm | (0.7) | (0.9) | (6.3) | (2.7) | (3.6) | (5.0) | (0.8) | (0.5) | (1.7) | (0.4) | (0.6) | (1.4) | (1.8) | (0.8) | (0.2) | (2.5) | (0.01) | (0.02) | (0.5) | (0.5) |
| t = 48 | 2.4 | 4.0 | 23.7 | 37.0 | 56.0 | 39.5 | 1.9 | 2.8 | 3.1 | 2.9 | 5.1 | 2.5 | 3.9 | 4.8 | 3.9 | 6.6 | 0.06 | 0.03 | 7.1 | 6.3 |
| 2 pm | (0.3) | (0.5) | (0.5) | (5.3) | (2.0) | (5.7) | (0.6) | (0.1) | (0.3) | (0.2) | (0.1) | (1.2) | (0.7) | (2.0) | (1.3) | (0.4) | (0.00) | (0.01) | (0.9) | (0.6) |
| t = 96 | 2.2 | 2.9 | 13.3 | 26.9 | 61.9 | 52.4 | 1.2 | 2.0 | 2.5 | 2.1 | 6.7 | 3.9 | 2.4 | 2.8 | 9.7 | 6.9 | 0.08 | 0.05 | 4.3 | 6.7 |
| 2 pm | (0.4) | (0.6) | (2.6) | (3.5) | (3.0) | (5.3) | (0.5) | (0.3) | (0.3) | (0.2) | (0.7) | (1.9) | (0.7) | (1.1) | (0.4) | (1.3) | (0.01) | (0.02) | (0.2) | (1.1) |

(continued)

**Table 3.** (continued)

| Sample time | HC | | WE/SE | | TG/FFA | | ALC | | ST | | Chl *a* | | Ampl | | PL | | Chl *a*/Neut | | Neut/Polar | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| hr of day | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. |
| (B) = Exposure initiated at 6 pm | | | | | | | | | | | | | | | | | | | | |
| t = 0 | 4.9 | | 36.3 | | 36.8 | | 2.4 | | 3.2 | | 3.6 | | 5.6 | | 7.2 | | 0.04 | | 5.2 | |
| 6 pm | (0.7) | | (1.3) | | (2.2) | | (0.4) | | (0.3) | | (0.9) | | (1.0) | | (0.8) | | (0.01) | | (0.3) | |
| t = 2 | 8.3 | 1.7 | 19.6 | 35.3 | 54.1 | 52.0 | 0.4 | 2.8 | 1.6 | 2.1 | 3.6 | 1.8 | 4.2 | 2.5 | 8.2 | 1.7 | 0.04 | 0.02 | 5.6 | 15.6 |
| 8 pm | (2.9) | (0.3) | (3.4) | (3.0) | (8.8) | (3.4) | (0.4) | (0.4) | (0.2) | (0.1) | (0.9) | (0.2) | (0.8) | (0.3) | (1.2) | (0.2) | (0.01) | (0.00) | (1.0) | (0.9) |
| t = 24 | 4.1 | 2.6 | 23.3 | 33.3 | 53.6 | 38.6 | 0.6 | 3.0 | 2.2 | 3.1 | 5.5 | 3.9 | 3.3 | 4.0 | 7.5 | 11.4 | 0.07 | 0.03 | 5.2 | 7.0 |
| 6 pm | (0.9) | (0.5) | (3.8) | (3.1) | (3.0) | (2.4) | (0.3) | (0.6) | (0.2) | (0.7) | (0.6) | (0.8) | (0.8) | (1.0) | (0.5) | (4.5) | (0.01) | (0.01) | (0.2) | (3.5) |
| t = 48 | 1.4 | 2.9 | 34.6 | 49.2 | 49.5 | 33.7 | 3.9 | 4.5 | 2.2 | 1.4 | 1.7 | 1.6 | 3.8 | 4.1 | 2.9 | 2.5 | 0.02 | 0.02 | 11.2 | 11.0 |
| 6 pm | (0.3) | (0.4) | (5.2) | (2.6) | (6.3) | (3.8) | (0.7) | (1.1) | (0.6) | (0.6) | (0.4) | (0.6) | (0.6) | (0.9) | (0.7) | (0.4) | (0.00) | (0.01) | (1.1) | (0.2) |
| t = 96 | 2.6 | 4.4 | 26.0 | 23.3 | 57.1 | 61.2 | 2.4 | 1.8 | 3.0 | 1.3 | 3.9 | 4.9 | 2.5 | 1.0 | 2.6 | 2.0 | 0.04 | 0.05 | 10.3 | 12.2 |
| 6 pm | (0.5) | (0.2) | (2.1) | (2.0) | (2.2) | (3.5) | (0.3) | (0.1) | (0.0) | (0.2) | (1.2) | (0.7) | (1.3) | (0.2) | (0.3) | (0.3) | (0.01) | (0.01) | (0.8) | (2.0) |

**Table 4.** Lipid class composition of *Melosira varians*. Control and exposure cultures. Growth conditions 16:8 h L/D at 20°. 2 pm samples were in the 11th hour of the light cycle; 6 pm samples were in the 15th hour of the light cycle. Sample times are annotated with actual time of day. Selected ratios and % composition presented as averages and standard error ( )

| Sample time | HC | | WE/SE | | TG/FFA | | ALC | | ST | | Chl *a* | | Ampl | | PL | | Chl *a*/Neut | | Neut/Polar | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| hr of day | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. |
| (A) = Exposure initiated at 2 pm | | | | | | | | | | | | | | | | | | | | |
| t = 0 | 0.7 | | 3.9 | | 65.4 | | 0.3 | | 4.1 | | 11.5 | | 7.8 | | 6.3 | | 0.16 | | 3.1 | |
| 2 pm | (0.1) | | (0.3) | | (1.4) | | (0.2) | | (0.5) | | (1.6) | | (1.3) | | (1.3) | | (0.03) | | (0.3) | |
| t = 2 | 0.9 | 1.5 | 4.7 | 1.0 | 63.5 | 81.9 | 0.5 | 0.0 | 5.9 | 3.0 | 10.2 | 5.4 | 5.6 | 3.5 | 8.8 | 3.7 | 0.14 | 0.06 | 3.2 | 9.9 |
| 4 pm | (0.1) | (0.5) | (1.6) | (0.3) | (3.0) | (5.6) | (0.3) | (0.0) | (0.5) | (1.6) | (3.8) | (1.5) | (1.0) | (1.2) | (3.2) | (1.4) | (0.06) | (0.02) | (0.4) | (3.7) |
| t = 24 | 4.1 | 5.7 | 4.1 | 5.1 | 58.7 | 63.9 | 0.4 | 0.0 | 5.1 | 3.8 | 10.3 | 4.5 | 4.9 | 8.6 | 12.3 | 8.4 | 0.14 | 0.06 | 2.7 | 5.7 |
| 2 pm | (1.4) | (1.6) | (1.0) | (0.4) | (2.7) | (8.3) | (0.4) | (0.0) | (0.3) | (1.1) | (1.4) | (3.0) | (0.5) | (3.6) | (2.5) | (2.4) | (0.02) | (0.04) | (0.2) | (2.0) |
| t = 48 | 1.5 | 2.2 | 6.2 | 1.1 | 61.9 | 87.4 | 0.4 | 0.0 | 3.5 | 1.8 | 10.8 | 2.2 | 6.7 | 3.3 | 9.1 | 2.0 | 0.17 | 0.02 | 3.2 | 13.2 |
| 2 pm | (0.5) | (1.3) | (1.9) | (0.7) | (7.[illegible]) | (1.9) | (0.2) | (0.0) | (0.8) | (0.3) | (4.3) | (1.3) | (1.6) | (0.3) | (3.1) | (0.3) | (0.08) | (0.01) | (0.7) | (2.1) |

(continued)

**Table 4.** (continued)

| Sample time | HC | | WE/SE | | TG/FFA | | ALC | | ST | | Chl *a* | | Ampl | | PL | | Chl *a*/Neut | | Neut/Polar | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| hr of day | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. |
| (B) = Exposure initiated at 6 pm | | | | | | | | | | | | | | | | | | | | |
| t = 0 | 1.6 | | 3.6 | | 68.7 | | 0.6 | | 3.9 | | 6.0 | | 7.4 | | 8.1 | | 0.08 | | 4.3 | |
| 6 pm | (0.6) | | (1.2) | | (5.1) | | (0.3) | | (0.4) | | (0.9) | | (1.6) | | (2.0) | | (0.01) | | (0.7) | |
| t = 2 | 1.0 | 2.2 | 1.6 | 4.9 | 80.7 | 65.6 | 0.0 | 0.4 | 2.7 | 7.6 | 5.6 | 7.4 | 5.9 | 9.3 | 2.4 | 2.7 | 0.07 | 0.09 | 6.4 | 4.2 |
| 8 pm | (0.4) | (0.3) | (0.5) | (0.6) | (2.1) | (5.0) | (0.0) | (0.4) | (0.7) | (3.6) | (0.8) | (0.7) | (2.7) | (1.2) | (0.1) | (0.3) | (0.01) | (0.01) | (1.0) | (0.3) |
| t = 24 | 1.3 | 3.0 | 7.6 | 3.8 | 66.3 | 65.6 | 1.9 | 0.4 | 2.8 | 3.0 | 5.3 | 4.0 | 8.0 | 5.9 | 6.7 | 14.2 | 0.07 | 0.05 | 4.2 | 3.3 |
| 6 pm | (0.3) | (0.5) | (1.5) | (0.6) | (1.9) | (3.2) | (0.8) | (0.3) | (0.5) | (0.4) | (0.1) | (1.1) | (0.9) | (0.9) | (1.6) | (1.3) | (0.00) | (0.02) | (0.7) | (0.5) |
| t = 48 | 1.3 | 3.4 | 3.2 | 3.4 | 72.2 | 62.9 | 0.5 | 0.0 | 2.8 | 2.4 | 6.9 | 5.6 | 4.7 | 6.2 | 8.4 | 16.1 | 0.09 | 0.08 | 4.6 | 2.6 |
| 6 pm | (0.1) | (0.1) | (0.4) | (0.1) | (4.5) | (0.0) | (0.3) | (0.0) | (0.1) | (0.3) | (1.8) | (0.6) | (0.8) | (0.3) | (2.7) | (1.2) | (0.03) | (0.01) | (0.9) | (0.0) |

**Table 5.** Lipid class composition of *Melosira varians*. Control and exposure cultures. Growth conditions 16:8 h L/D at 15°. 2 pm samples were in the 11th hour of the light cycle; 6 pm samples were in the 15th hour of the light cycle. Sample times are annotated with actual time of day. Selected ratios and % composition presented as averages and standard error ( )

| Sample time | HC | | WE/SE | | TG/FFA | | ALC | | ST | | Chl *a* | | Ampl | | PL | | Chl *a*/Neut | | Neut/Polar | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| hr of day | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. |
| (A) = Exposure initiated at 2 pm | | | | | | | | | | | | | | | | | | | | |
| t = 0 | 2.1 | | 16.1 | | 59.0 | | 2.2 | | 7.5 | | 6.2 | | 2.9 | | 4.1 | | 0.07 | | 6.8 | |
| 2 pm | (0.3) | | (0.9) | | (1.5) | | (0.2) | | (0.6) | | (1.9) | | (0.6) | | (0.7) | | (0.02) | | (0.6) | |
| t = 2 | 2.0 | 1.8 | 5.3 | 27.2 | 71.2 | 44.3 | 0.5 | 2.5 | 7.8 | 6.4 | 3.6 | 3.5 | 6.8 | 5.1 | 3.0 | 9.4 | 0.04 | 0.04 | 6.7 | 4.6 |
| 4 pm | (0.2) | (0.2) | (2.1) | (2.2) | (1.4) | (1.4) | (0.4) | (0.2) | (0.2) | (0.3) | (0.5) | (1.0) | (1.4) | (0.4) | (0.2) | (1.8) | (0.01) | (0.01) | (0.7) | (0.2) |
| t = 24 | 3.2 | 1.9 | 23.4 | 21.5 | 48.7 | 48.7 | 0.8 | 3.0 | 4.9 | 7.6 | 6.5 | 5.6 | 8.7 | 4.0 | 3.9 | 7.8 | 0.08 | 0.07 | 4.3 | 4.8 |
| 2 pm | (0.2) | (0.3) | (0.5) | (1.4) | (1.3) | (1.2) | (0.5) | (0.2) | (1.4) | (0.6) | (1.3) | (0.8) | (3.2) | (0.6) | (1.7) | (0.7) | (0.02) | (0.01) | (0.3) | (0.3) |
| t = 48 | 3.1 | 2.8 | 35.7 | 27.3 | 37.9 | 41.7 | 4.2 | 4.3 | 5.4 | 8.0 | 4.6 | 6.1 | 4.8 | 4.7 | 4.3 | 5.2 | 0.05 | 0.07 | 6.5 | 5.3 |
| 2 pm | (0.4) | (0.4) | (7.0) | (1.4) | (4.3) | (1.6) | (0.8) | (0.8) | (0.8) | (1.0) | (1.4) | (0.9) | (1.3) | (1.0) | (1.3) | (0.2) | (0.02) | (0.01) | (0.8) | (0.3) |
| t = 96 | 2.2 | 1.7 | 13.0 | 11.3 | 65.6 | 67.1 | 1.6 | 1.2 | 4.1 | 5.0 | 5.1 | 4.4 | 2.9 | 3.2 | 5.7 | 6.0 | 0.06 | 0.05 | 6.6 | 6.6 |
| 2 pm | (0.5) | (0.2) | (2.2) | (1.7) | (4.1) | (3.3) | (0.2) | (0.5) | (0.4) | (0.7) | (1.3) | (1.9) | (0.8) | (0.9) | (1.2) | (1.2) | (0.02) | (0.02) | (0.8) | (0.8) |

(continued)

**Table 5.** (continued)

| Sample time | HC | | WE/SE | | TG/FFA | | ALC | | ST | | Chl a | | Ampl | | PL | | Chl a/Neut | | Neut/Polar | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| hr of day | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. |
| B) = Exposure initiated at 6 pm | | | | | | | | | | | | | | | | | | | | |
| t = 0 | 1.6 | | 17.2 | | 62.5 | | 2.2 | | 6.6 | | 2.6 | | 4.0 | | 3.4 | | 0.03 | | 9.4 | |
| 6 pm | (0.1) | | (3.0) | | (4.0) | | (0.2) | | '0.3) | | (0.6) | | (0.4) | | (0.4) | | (0.01) | | (0.9) | |
| t = 2 | 3.2 | 1.2 | 2.2 | 10.7 | 68.3 | 65.4 | 2.8 | 1.2 | 9.1 | 7.8 | 2.6 | 2.3 | 7.3 | 6.2 | 4.5 | 5.0 | 0.03 | 0.03 | 6.4 | 6.4 |
| 8 pm | (0.1) | (0.1) | (0.1) | (0.6) | (2.5) | (1.4) | (0.1) | (0.5) | (0.6) | (0.7) | (0.3) | (0.5) | (0.9) | (0.5) | (1.4) | (1.0) | (0.00) | (0.01) | (1.1) | (0.0) |
| t = 24 | 2.7 | 2.6 | 18.7 | 16.5 | 46.4 | 53.2 | 1.8 | 3.3 | 8.2 | 8.7 | 5.3 | 4.1 | 6.8 | 6.3 | 10.1 | 5.4 | 0.07 | 0.05 | 3.8 | 5.6 |
| 6 pm | (0.6) | (0.6) | (0.6) | (8.2) | (3.7) | (7.1) | (0.6) | (0.6) | (1.2) | (1.6) | (0.8) | (0.9) | (2.5) | (2.6) | (1.0) | (2.2) | (0.01) | (0.01) | (0.7) | (0.7) |
| t = 48 | 2.6 | 1.6 | 23.7 | 32.7 | 48.8 | 46.5 | 3.2 | 2.9 | 7.5 | 4.8 | 4.0 | 5.1 | 4.2 | 2.3 | 6.1 | 4.1 | 0.05 | 0.06 | 6.5 | 7.9 |
| 6 pm | (0.2) | (0.3) | (2.0) | (3.4) | (3.6) | (2.7) | (0.4) | (0.3) | (1.1) | (0.4) | (0.8) | (1.8) | (1.0) | (0.9) | (1.4) | (0.9) | (0.01) | (0.02) | (1.1) | (1.0) |
| t = 96 | 1.9 | 1.5 | 24.0 | 12.6 | 56.4 | 62.0 | 1.4 | 0.7 | 5.2 | 6.0 | 3.2 | 6.6 | 2.9 | 2.3 | 5.0 | 8.1 | 0.04 | 0.08 | 9.2 | 4.9 |
| 6 pm | (0.2) | (0.3) | (6.6) | (2.3) | (4.7) | (2.4) | (0.1) | (0.4) | (0.7) | (0.5) | (0.4) | (0.9) | (0.1) | (0.9) | (1.7) | (0.3) | (0.01) | (0.01) | (2.4) | (0.2) |

## RESULTS

### *Cyclotella Lipid Class Composition*

Detailed lipid class composition and selected ratios of lipid classes in *Cyclotella* are presented in Tables 2-3. These data demonstrate that lipid class composition of control cultures is considerably different at the two times of day sampled and that temperature may alter lipid class composition. Control data from this experiment and the experiment reported in the following paper were pooled and selected parameters (chlorophyll, triacylglycerols, polar lipid, and the neutral/polar lipid ratio) graphed to determine variation with time. These results are presented in Figure 1. At 20°C (Table 2 and Figure 1) control cultures are characterized by a higher chlorophyll content than when cells are maintained at 15°C (Table 3). Average neutral/polar lipid ratios are higher at 15°C. Several fractions of neutral lipids vary considerably with temperature in this diatom. At 20°C, the predominant neutral lipid class is triacylglycerol, comprising approximately 70% of the total lipid. At 15°C, triacylglycerols and a class consisting of wax and sterol esters are both abundant and present in approximately equal proportions.

### *Melosira Lipid Class Composition*

Detailed lipid class composition ot *Melosira* is presented in Tables 4-5 and variations in chlorophyll, triacylglycerol, polar lipid, and the neutral/polar lipid ratio of control cells are presented in Figure 2. Like *Cyclotella*, cultures of *Melosira* grown at 20°C have different lipid profiles when compared with cells cultured at 15°C. At 20°C, chlorophyll values are considerably higher. Chlorophyll reaches a peak between the 11th and 13th hours of light at 20°C and is also higher in the 11th hour of light at 15°C. Neutral to polar lipid ratios rise steadily through the last eight hours of the light period at 20°C, and reach a maximum prior to the onset of dark at 15°C. Cultures maintained at 15°C, like *Cyclotella*, have higher percentages of wax/sterol esters (Table 5). In addition neutral/polar lipid ratios are approximately doubled at this temperature.

### *Total Extractable Lipid (TEL) as a Function of Light/Dark Cycle*

TEL data from several diel studies as well as values from this paper were pooled and plotted as a function of hour in the 16:8 h L/D cycle. These data are presented in Figures 3-4. The diel pattern of TEL in *C. meneghiniana* (Figure 3) demonstrates that there are cyclical variations in lipid content at 20°C. Most noticeably, there are peaks in the early (first 8 hours) and late (second 8 hours) light periods as well as in the dark period, although the general trend is an increase in the late light and dark periods, with a reduction in total lipid toward the end of the dark period. In contrast, at 15°C, TEL content is both uniform and low.

The TEL distribution pattern over a diel cycle is quite different in *Melosira* (Figure 4). TEL content is quite uniform for the first 11 hours of light in *Melosira* cultured at 20°C. There is a reduction in the last four hours of the light period, and a bimodal distribution in the dark, which is followed by a return to levels found in the early light period. TEL content is more uniform and slightly lower at 15°C.

### *Changes in Lipid Classes During Exposure to 1,3,5-Trichlorobenzene*

Results of exposure experiments are presented in Tables 2-5 and in Figures 5-8. For ease of comparison, the percent change (exposed cells compared with control cultures) of the following three parameters have been graphed: Chlorophyll *a*, chlorophyll *a*/neutral lipid ratio, and neutral/polar lipid ratio. Exposure of *Cyclotella* to the chlorinated benzene resulted in few significant changes in the cultures (Figure 5). It appears that within 24 hours after the exposure was initiated, there was a reduction in chlorophyll and an increase in neutral lipid in the 11th hour exposure. However, there was recovery at 48 hours and values approached those of control cultures. The variability observed is within the range of the standard errors of the numbers (Table 2).

At 15°C, there was an overall reduction in chlorophyll in cultures exposed to the chlorinated benzene. The reduction appeared to be more pronounced when the exposure was initiated in the 11th hour of the light period (Figure 6). There was a general increase in the neutral/polar lipid ratio for both exposures (Figure 6, Table 3).

The response of *Melosira* to the chlorinated benzene was quite different. Exposure in the 11th hour of the light period at 20°C resulted in approximately an 80% reduction in chlorophyll content (Figure 7). There was also a reduction in chlorophyll in the 15th hour exposure. However, the magnitude of the reduction was considerably less (Table 4, Figure 7). During the 11th hour exposure, the neutral/polar lipid ratio was greater by a factor of ca. 2-3. In contrast, this ratio was reduced in the exposure initiated in the 15th hour of the light period. At 15°C, there was little variability in chlorophyll content or the neutral/polar lipid ratio (Figure 8, Table 5). Changes in ratios were within the range of variability of the replicates (Table 5).

## DISCUSSION

In general, lipophilic compounds having high octanol/water partition coefficients and low water solubilities tend to passively bioconcentrate. Accumulation of hydrophobic compounds appears to be related to lipid content (Canton *et al.* 1977; Clayton *et al.* 1977; Boyles 1980; Hutchinson *et al.* 1980). While lipid content may have an overall effect on bioaccumulation and food web transfer (indirect ecosystem effects), our results suggest that lipid class composition at the time of exposure may alter the short-term direct effects of the toxicant on individual phytoplankton taxa.

Results presented here demonstrate that *Melosira varians* experienced more changes when exposed to 1,3,5-trichlorobenzene than *Cyclotella meneghiniana*. The small effects observed in *Cyclotella* are in agreement with previous studies which demonstrated that of the three trichlorobenzene isomers, the 1,3,5 isomer had the least effect (Sicko-Goad *et al.* 1989a-d). These data might be explained in view of the physiological ecology of the organisms, diel total extractable lipid content, and lipid class composition patterns.

*Cyclotella meneghiniana* demonstrates a strong preference for long days (i.e. 20:4 h L/D) for optimal growth (Sicko-Goad and Andresen 1991). Growth occurs at 16:8 h L/D, but it is not optimal. A reduction in temperature from 20°C to 15°C enhances growth at 16:8, and these more rapidly growing cells are characterized by a reduced average diel neutral/polar lipid ratio (Sicko-Goad and Andresen, unpublished observation), and a reduced triacylglycerol/polar lipid ratio.

*Melosira varians*, on the other hand, is able to tolerate a wide variety of light regimes (12:12, 16:8, 20:4 h L/D), although growth is somewhat more rapid and sustained for longer periods of time under a 12:12 h L/D regime. Lipid class analyses under a variety of light regimes suggests that of the two light and temperature regimes utilized in this experiment, the 16:8 h regime at 20°C results in higher average diel concentrations of chlorophyll a and polar lipids. Since more effects occurred in *Cyclotella* at 15°C and in *Melosira* at 20°C, it is apparent that more rapidly growing cells are more susceptible to short term effects of lipophilic toxicants.

The results of this experiment and our previous experiments with *Cyc lla* (Sicko-Goad *et al.* 1989a-d) may best be explained in terms of the ratio of triacylglycerol to ar lipids. For the four previous exposure experiments, we found exposure to both 1,2,3- and 1,3,5-trichlorobenzene resulted in significant changes in fatty acid composition and morphological compartments early in the sampling scheme. That is, most changes occurred with the first 24 hours of exposure. Both of these experiments were initiated between the 6th and 7th hours of the light period, when triacylglycerol is low and polar lipids are high. Experiments with 1,2,4-trichlorobenzene and pentachlorobenzene were initiated between the 10th and 11th hours of the light period, when triacylglycerols are approximately doubled in the cells. Effects of the latter compounds were

observed in greater numbers in later sampling times (5 days). In the present experiment, changes were observed in lipid class composition (i.e. increase in triacylglycerols, reduction in chlorophyll, increase in the neutral/polar lipid ratio) when the exposure was initiated in the 11th hour of the light cycle at 20°C and in both exposures at 15°C. Cultures grown under these conditions generally have a lower concentration of triacylglycerols and lower ratios of TG/polar lipids when compared with samples taken in the 15th hour of light. Although some changes were observed, 1,3,5 trichlorobenzene in concentrations near that of water solubility does not have much overall effect on *Cyclotella meneghiniana*.

The pattern of effect appears to be somewhat similar, though more pronounced in *Melosira varians*, where short term effects were observed in the 11th hour exposure at 20°C. Diel lipid class composition patterns of the two diatoms show remarkable similarities. Although in *Melosira* chlorophyll concentration is higher in the 11th hour than in the 15th hour, both diatoms experience increases in chlorophyll concentration at the time exposure was initiated. For *Melosira*, the increase is observed between the 11th and 13th hours of the light period, whereas in *Cyclotella*, increases are observed between the 11th and 15th hours. Triacylglycerol is also lower in *Melosira* in the 11th hour of the light period. Polar lipids are at their maximum at this time, and the neutral/polar lipid ratio rises steadily in the latter part of the light period. Consequently, it appears that at least some short term effects of 1,3,5-trichlorobenzene are more pronounced when exposure occurs when chlorophyll synthesis is occurring, polar lipids are generally higher, and the ratio of triacylglycerol to polar lipid is reduced.

Relatively little is known about photoperiodic changes in lipid content and composition in phytoplankton. Shifrin and Chisholm (1981) demonstrated that changes in total lipids over a diel cycle were directly related to cell growth and reproduction. Similarly, Rivkin (1985) reported diatoms utilize lipids and low molecular weight polymers for respiration and/or protein synthesis at night. In a more recent study, Sukenik and Carmeli (1991) demonstrated that in a Eustigmatophycean alga, *Nannochloropsis*, neutral lipids such as triacylglycerol were synthesized and accumulated in the light period and rapidly turned over in the dark. These authors demonstrated that on a 12:12 h L/D cycle, triacylglycerols increased more than threefold in a period of six hours preceding the onset of the dark period. These studies demonstrate that large variations in lipid content and composition occur as part of a natural diel cycle in a wide variety of phytoplankton taxa.

The short-term changes that occurred in *Cyclotella* were minor and some recovery was evident at 48 hours. Rapid recovery from short-term or sublethal toxicant doses is not uncommon (Soto *et al.* 1977; Sandmann and Boger 1980; Sicko-Goad 1982; Hardy *et al.* 1985). *Cyclotella* may also be less susceptible to 1,3,5 trichlorobenzene than *Melosira* because it extrudes chitin strands through the frustule. These chitin strands have at least one function of providing buoyancy for the cells (Smucker 1991) and may provide additional surface area for the adsorption of toxicants effectively reducing the concentration available to the cell. In addition to chitin strands, *C. meneghiniana* has been observed to produce copious mucilage during growth in culture (Sicko-Goad and Andresen unpublished observation). In the majority of the algae the role of mucilage is poorly documented, however speculations as to its varied roles abound (Boney 1981).

It has been suggested many times that environmental variables and physiological status of cells may play an important role in toxicity studies (Fisher *et al.* 1976; Conner and Mahanty 1979; Karydis and Fogg 1980; Sicko-Goad and Lazinsky 1986; Neumann *et al.* 1987). Our studies suggest that in addition to these factors, simple diel variations in metabolic patterns may also affect the outcome of toxicity studies. Extreme care should be taken in comparative toxicity bioassays of lipophilic compounds so that assays are always initiated at the same time of day to ensure uniform lipid content and composition.

## ACKNOWLEDGMENTS

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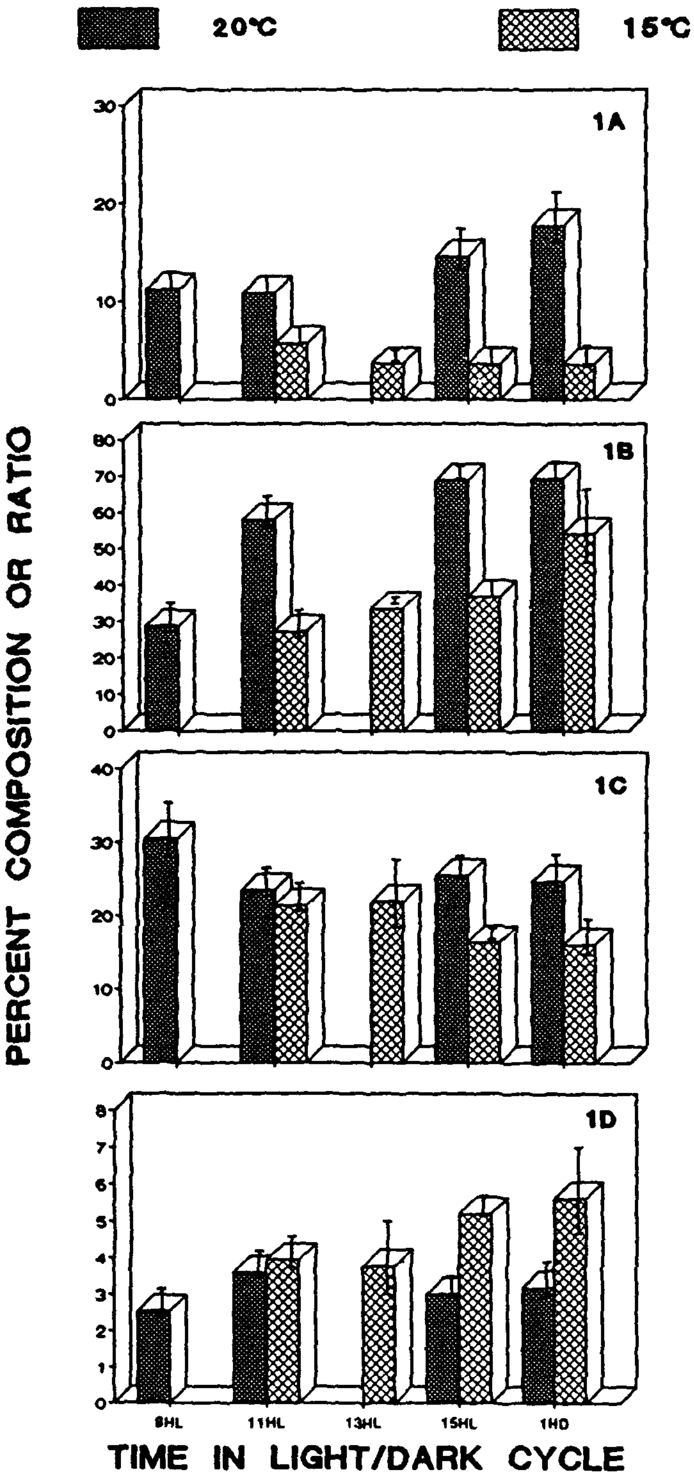
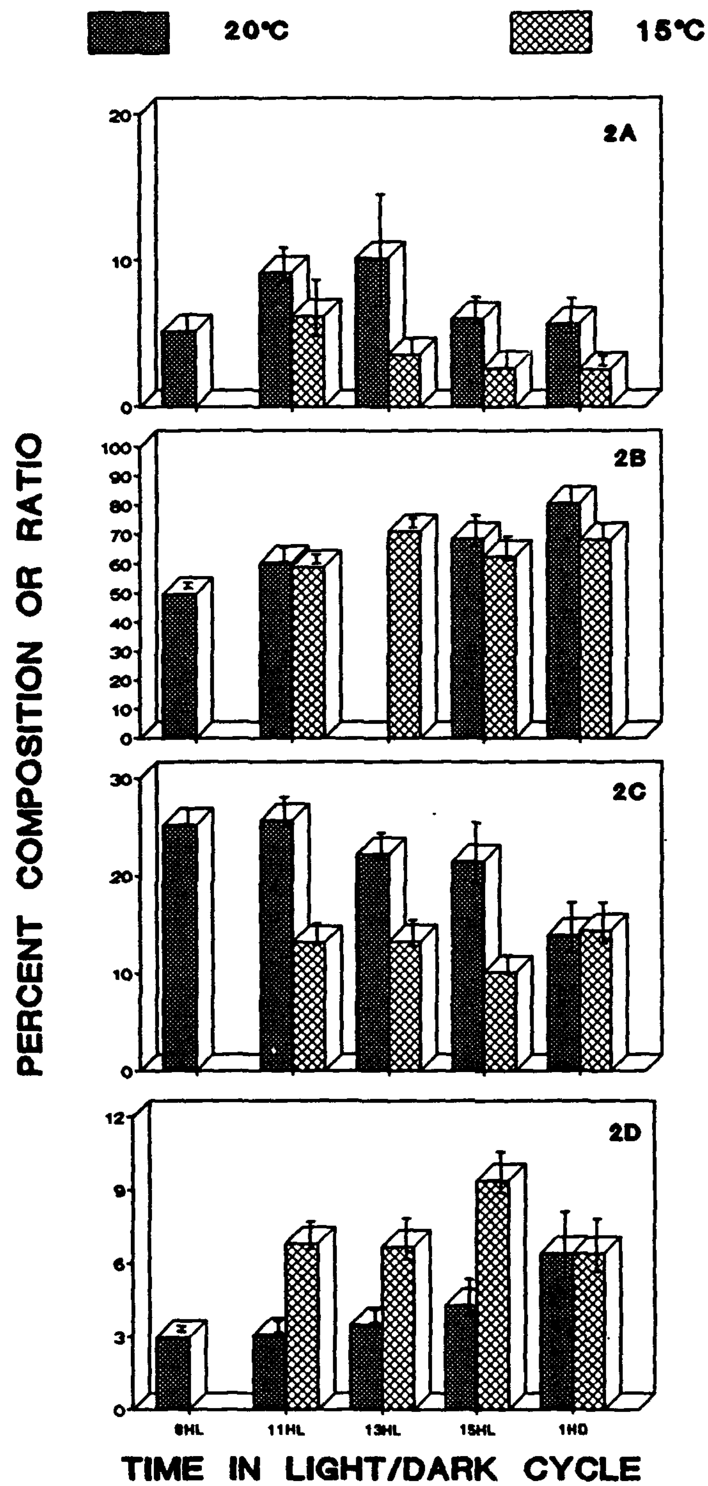
## LIST OF FIGURES



20°C
15°C
1A
1B
1C
1D
PERCENT COMPOSITION OR RATIO
9HL
11HL
13HL
15HL
1HD
TIME IN LIGHT/DARK CYCLE



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15°C
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2B
2C
2D
PERCENT COMPOSITION OR RATIO
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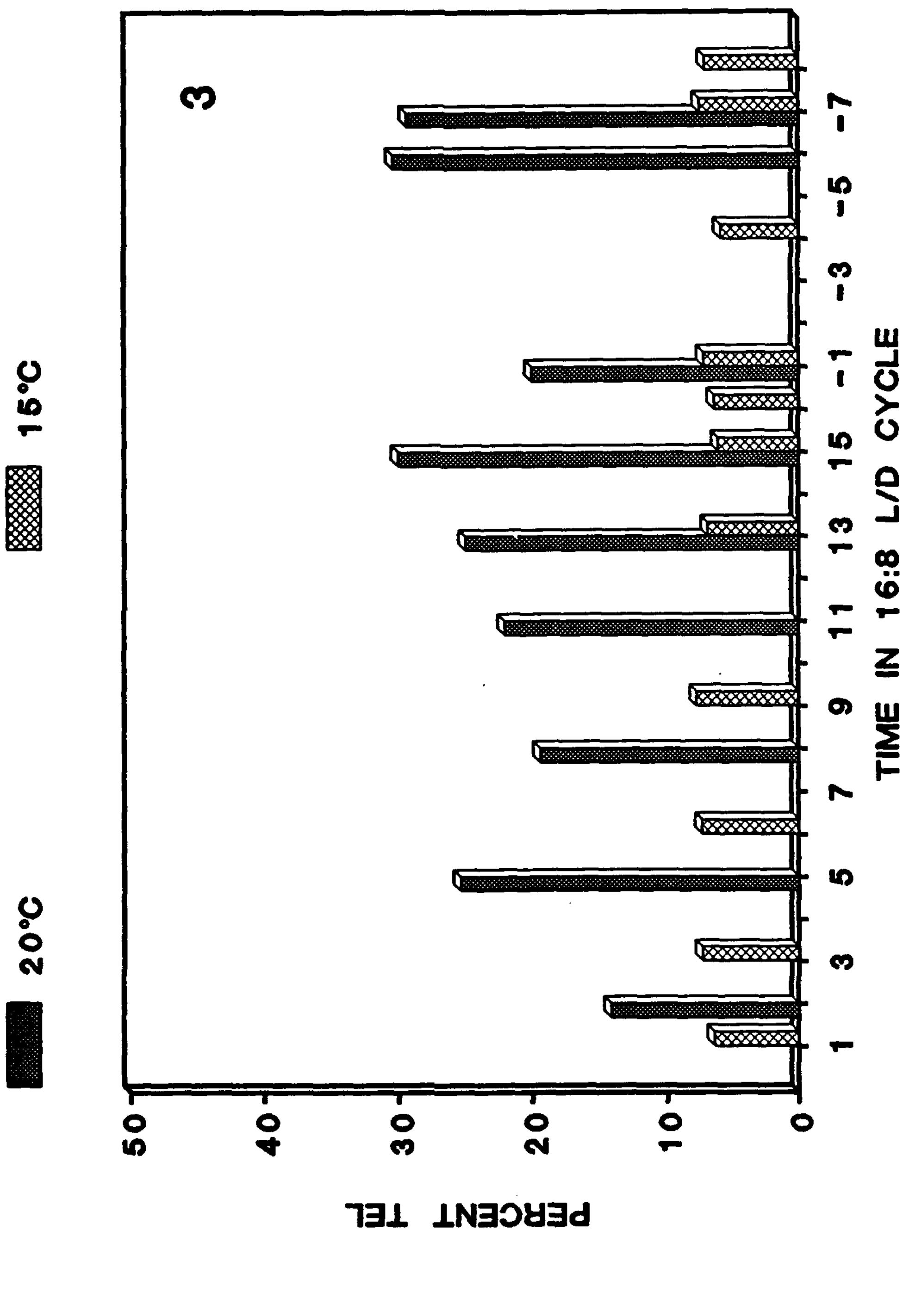
11HL
13HL
15HL
1HD
TIME IN LIGHT/DARK CYCLE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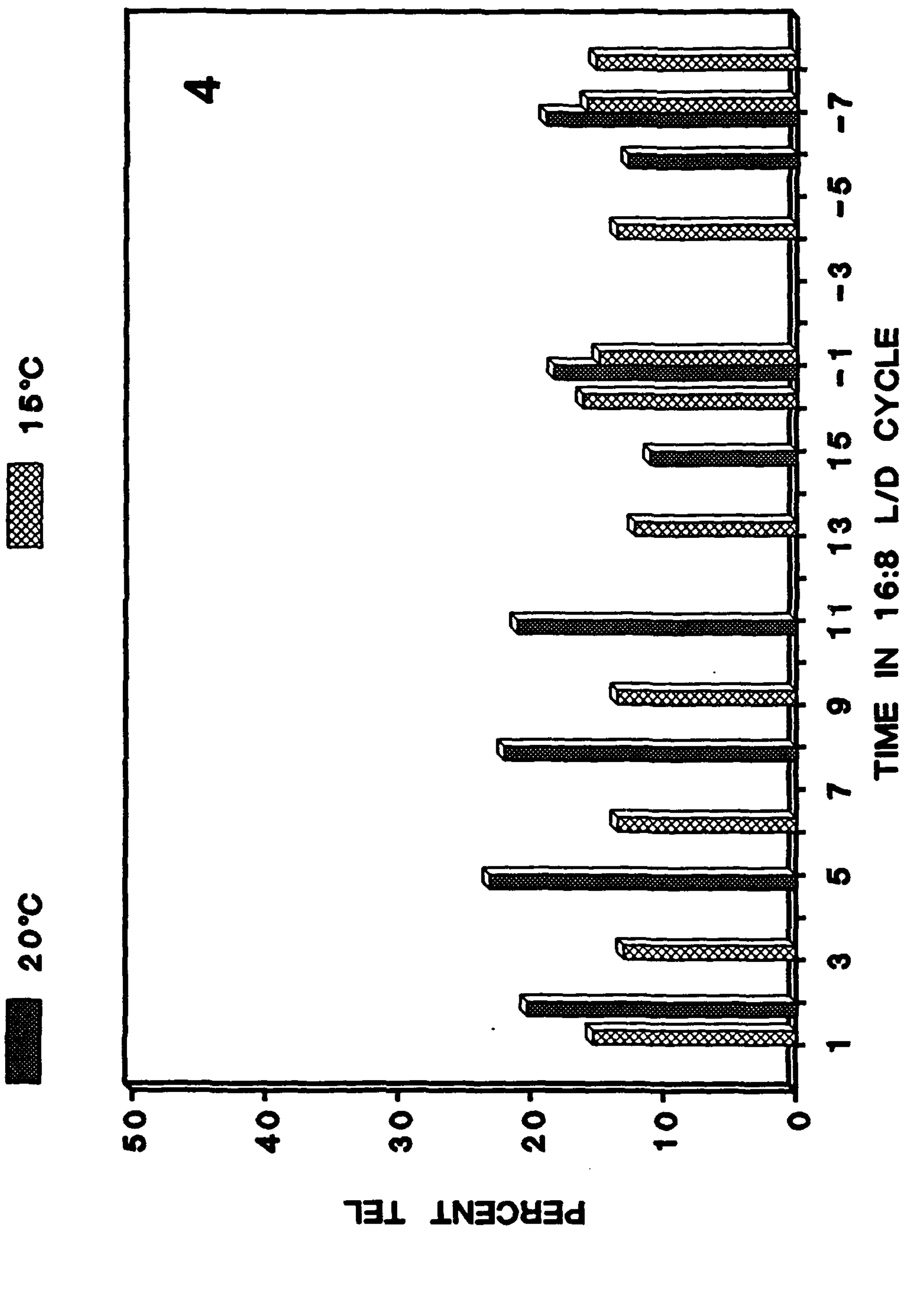
−7
TIME IN 16:8 L/D CYCLE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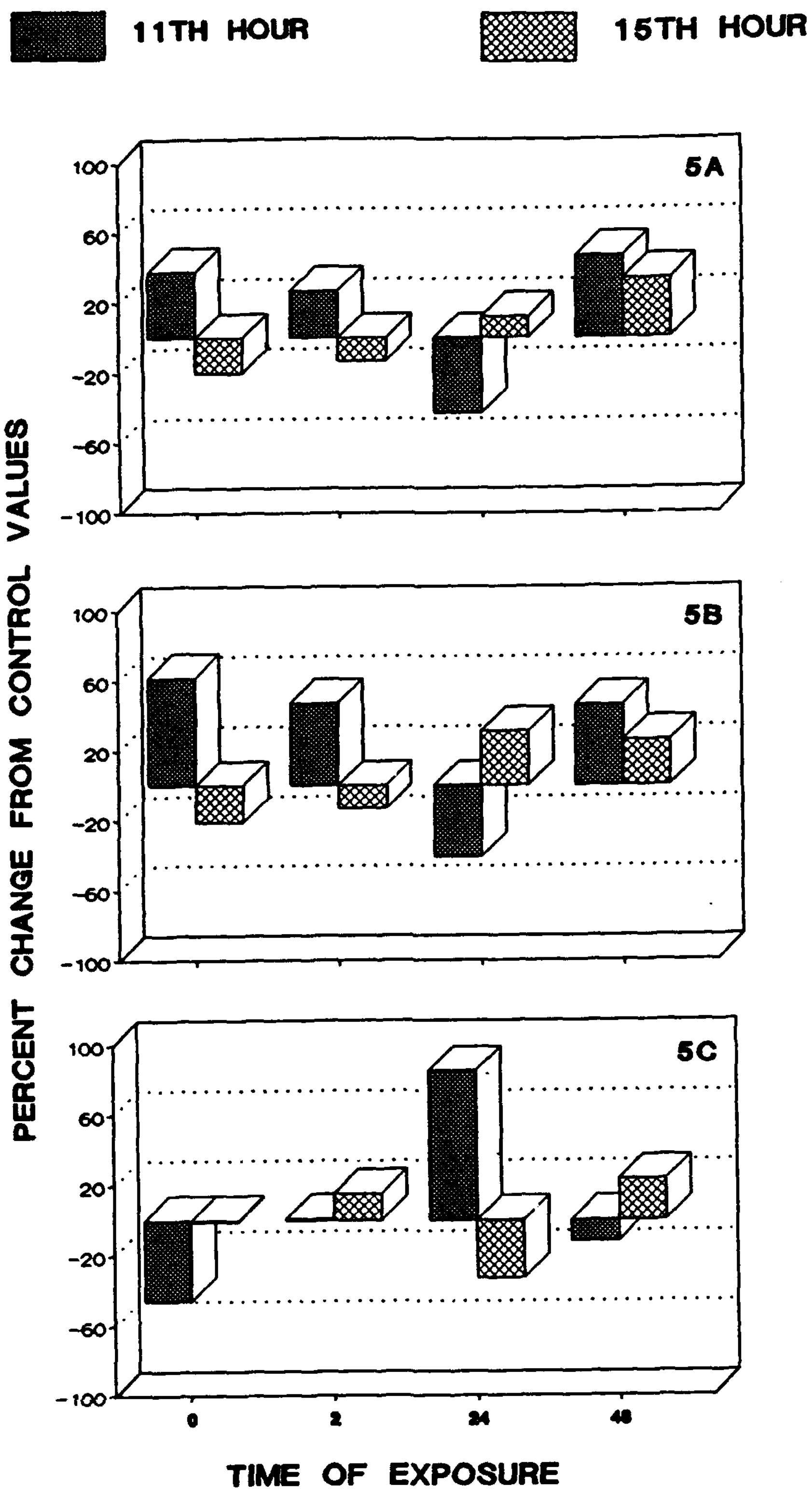

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15TH HOUR
PERCENT CHANGE FROM CONTROL VALUES
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5B
5C
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20
-20
-60
-100
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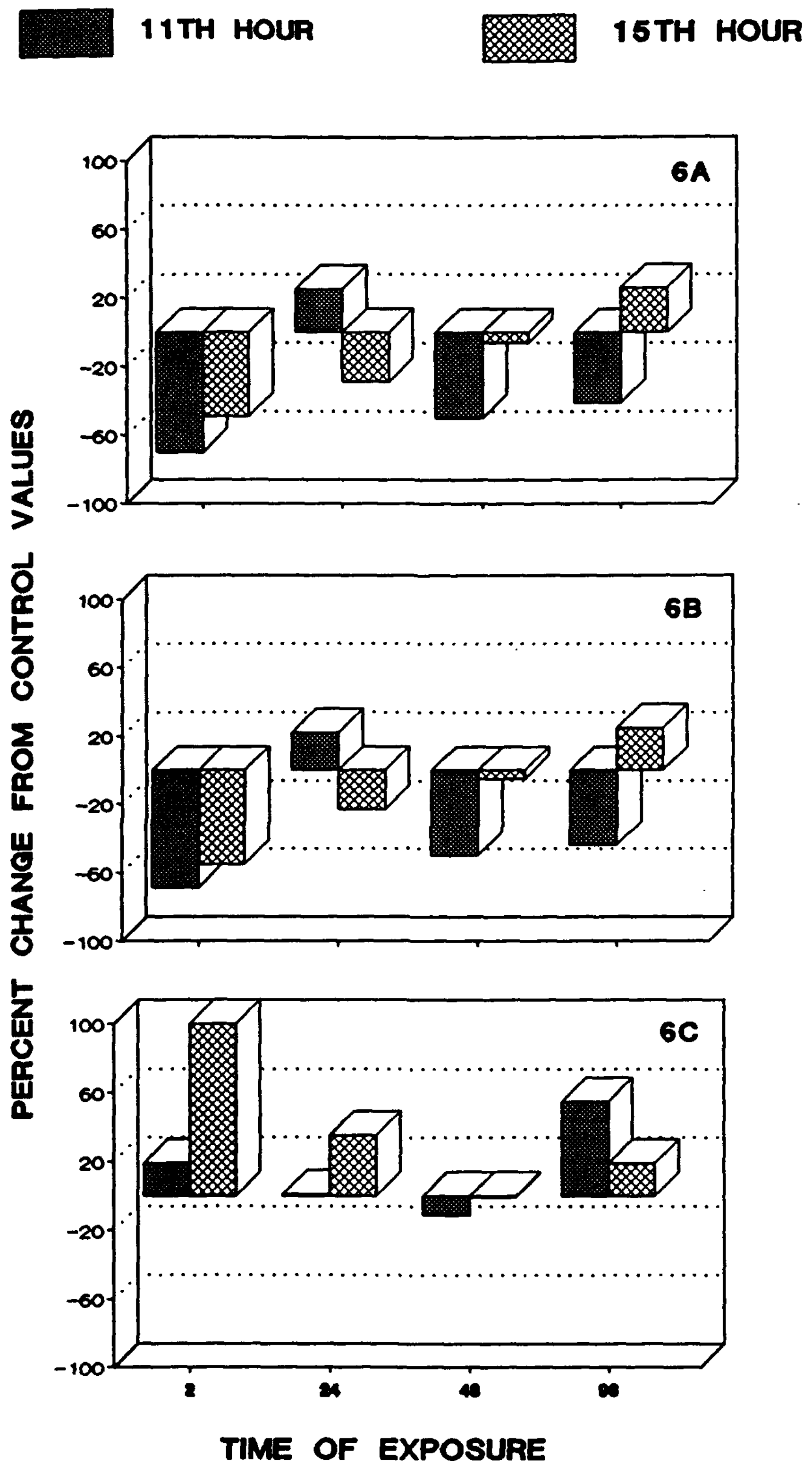
2
24
48
TIME OF EXPOSURE



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15TH HOUR
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6B
6C
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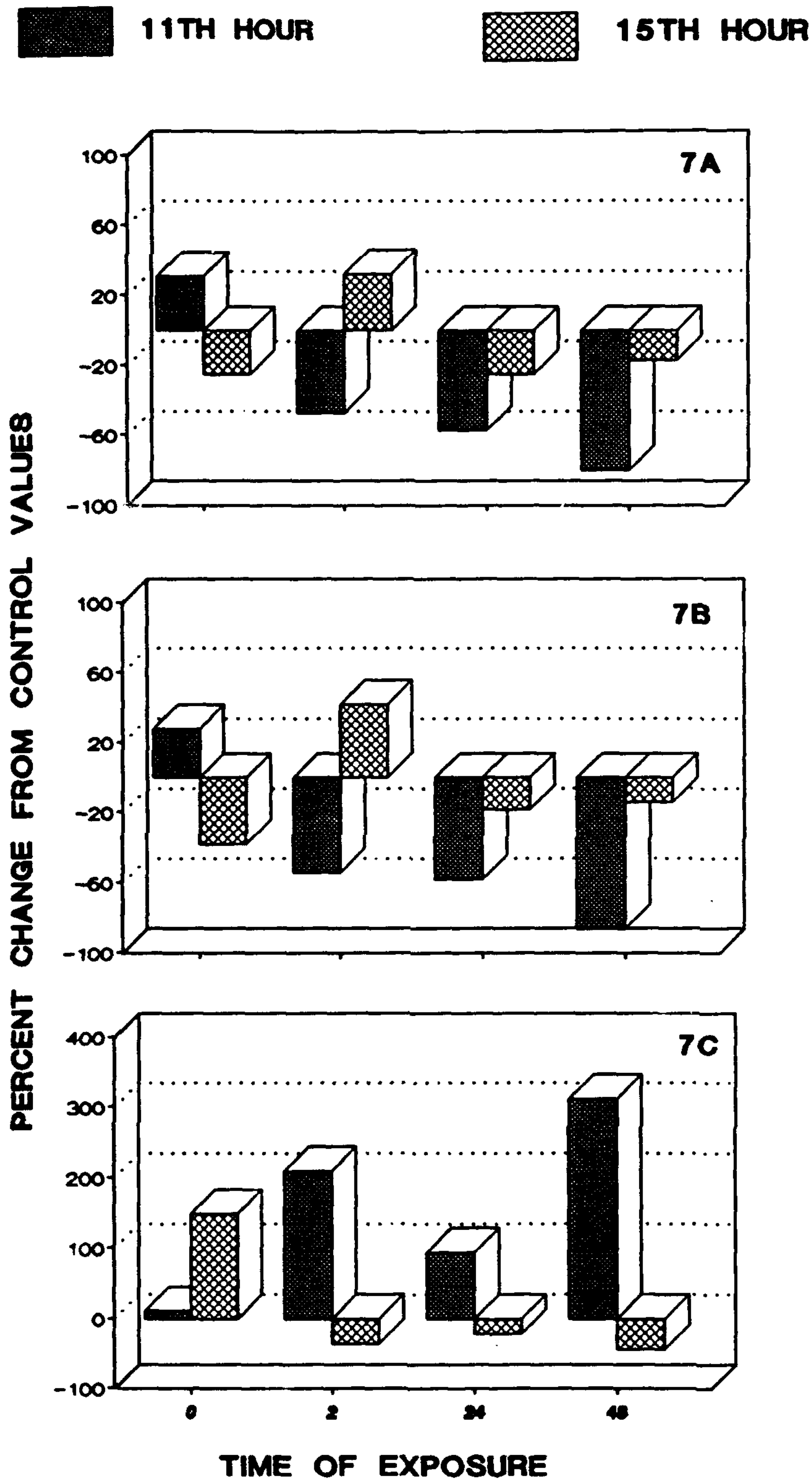
60
20
-20
-60
-100
PERCENT CHANGE FROM CONTROL VALUES
TIME OF EXPOSURE



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15TH HOUR
PERCENT CHANGE FROM CONTROL VALUES
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7B
7C
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20
-20
-60
-100
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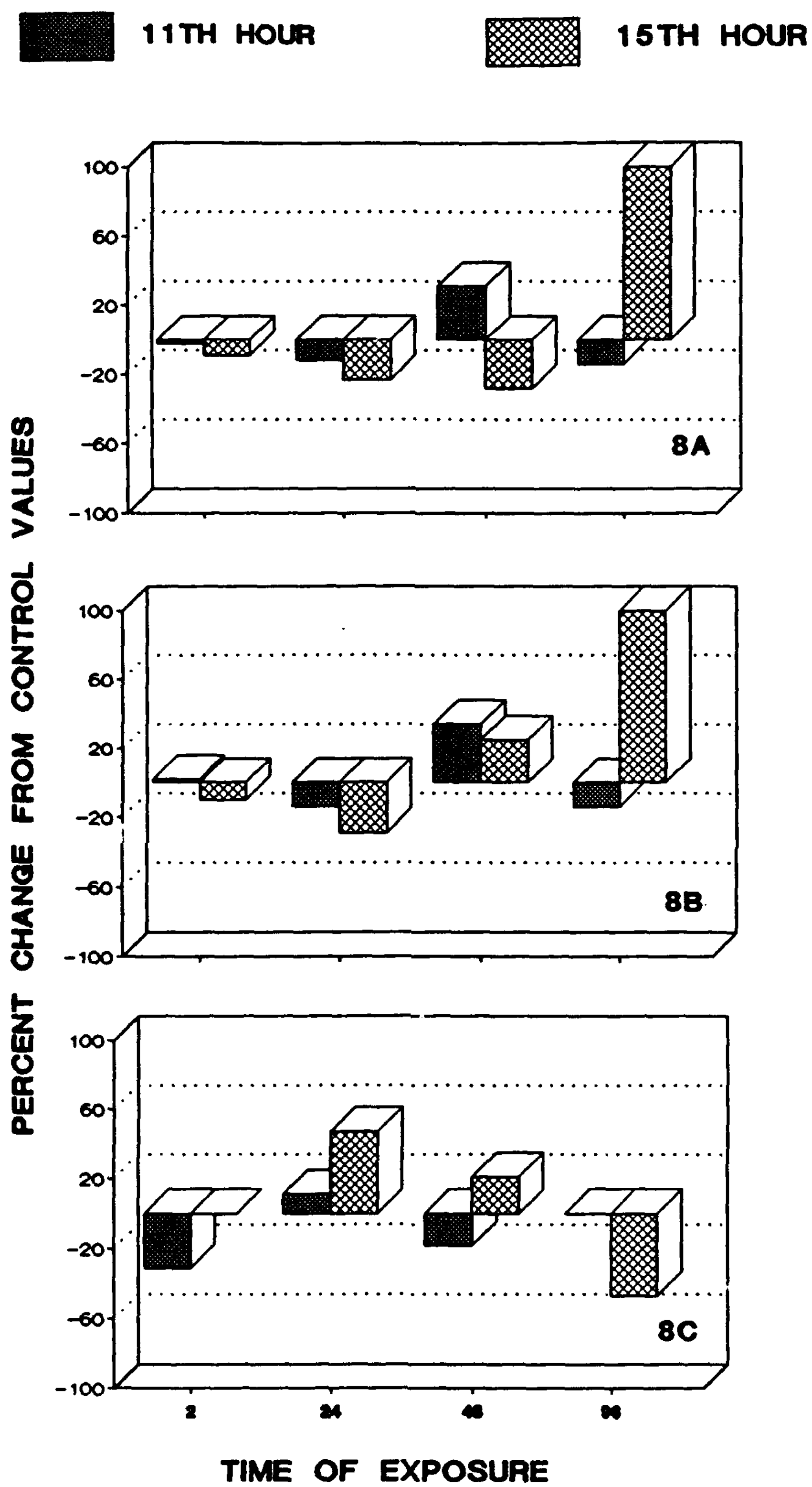
300
200
100
0
0
2
24
48
TIME OF EXPOSURE


11TH HOUR
15TH HOUR
PERCENT CHANGE FROM CONTROL VALUES
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60
20
-20
-60
-100
8A
8B
8C
TIME OF EXPOSURE

# EFFECT OF DIATOM LIPID COMPOSITION ON THE TOXICITY OF TRICHLOROBENZENE

## II. Long-term Effects of 1,2,3-trichlorobenzene

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**Abstract.** Exposures of four diatoms, *Cyclotella meneghiniana, Melosira italica, M. varians,* and *Synedra filiformis* to 0.3 ppm 1,2,3-trichlorobenzene were initiated at the 8th and 11th hours of the light period on a 16:8 h L/D cycle at 20°C. Cell counts, lipid content, and lipid class composition were monitored for 10 days. *Melosira italica* demonstrated the most long-term effects. Cell counts and chlorophyll *a* were greatly reduced on the 10th day when exposure was initiated in the 11th hour of the light period in *Synedra filiformis* and in the 8th hour of the light period in *Melosira varians*. The data demonstrate that more immediate effects occur when exposures are initiated during periods of high polar lipid content or when polar lipids are being synthesized. Long-term effects are observed when exposure initiation occurs during periods of high neutral lipid content and higher total extractable lipid. The results suggest that the response of diatoms to low levels of chlorinated benzenes is related to normal variation in diel lipid composition. These results are repeatable, they vary with species, and exposures initiated at different times of the day may produce quite different results.

## INTRODUCTION

Although lipophilic compounds such as organochlorines have been demonstrated to affect phytoplankton populations adversely (e.g. Galassi and Vighi 1981; Mahanty *et al.* 1983; Geyer *et al.* 1985; Wong *et al.* 1984; Halfon and Reggiani 1986), lower and/or chronic exposure of natural assemblages to a wide variety of toxicants often leads to increased phytoplankton abundance (North *et al.* 1964; Federle *et al.* 1979; Vargo *et al.* 1982). This is not to suggest that organic toxicants do not affect phytoplankton growth and survival. What has often been demonstrated is that chlorophyll synthesis (Conner and Mahanty 1979) and photosynthesis are reduced in these natural assemblages, often in combination with reduced predation rates (Vargo *et al.* 1982). What often occurs in impacted areas is the replacement of oligotrophic and mesotrophic algae with less desirable species of algae. In many areas of concern in the Laurentian Great Lakes phytoplankton standing stocks are high, but consist of blue-green and green algae, organisms which are low in food quality and inhibit zooplankton grazing (Arnold 1971; Richmond and Dodson 1983).

Diatoms appear to be particularly good food sources for many animals since they often have a high lipid content and high concentrations of eicosapentaenoic acid (Sicko-Goad *et al.* 1988; Volkman *et al.* 1989; Ahlgren *et al.* 1990). It is for this reason that we have continued toxicity studies with diatoms.

Our earlier studies (Sicko-Goad *et al.* 1989a-d; Sicko-Goad and Andresen 1992) demonstrated that exposures of diatoms to chlorinated benzenes for up to 5 days resulted in some detrimental changes in the cultures. However, it was not possible to predict from these studies whether or not the populations would recover from exposures. Some evidence of recovery was suggested. Therefore, we designed exposure experiments in which four diatom taxa were exposed to 1,2,3-trichlorobenzene for 10 days, with exposures initiated at 2 different times of days. The results of these experiments are presented here.

## MATERIALS AND METHODS

Most materials and methods have been described in the preceding paper (Sicko-Goad and Andresen 1992). For this experiment, however, two additional diatoms, *Melosira italica* (Ehrenb.) Kütz., and *Synedra filiformis* Grun. in Cl. & Grun., were used as experimental organisms. Culture conditions were identical to those previously described. The experiments were conducted in May and June, 1990 and consisted of an exposure of all four diatom taxa (individually) to 0.3 ppm 1,2,3-trichlorobenzene at 20°C. Several parameters were changed between this experiment and the preceding paper. First, no methanol was used as a carrier. Second, although the 16:8 h L/D cycle was maintained, the on-off timing was changed so that the lights came on three hours later in the day. Exposures were initiated at 2 pm and 5 pm in the afternoon, corresponding to the 8th and 11th hours of the light period. These changes were made in an attempt to ascertain if the periodicities observed in lipid composition were a function of entrainment with the light cycle, or if they were intrinsic with time of day. All samples were taken at the time of day the exposure was initiated (i.e., the 8th or 11th hour of the light period) and cultures were sampled on days 0, 1, 3, 5, 7 and 10.

The third change in experimental design was the addition of cell counts for all samples. Since the diatoms were growing rapidly and the experiment was 10 days in duration, changes in cell counts were expected. Concurrent with dry weight analyses, smaller volumes of culture medium (9mL) were withdrawn and placed in a tube containing paraformaldehyde and glutaraldehyde at final concentrations of 1% in 0.05M sodium cacodylate buffer at pH 7.2. Cell counts were performed with either a hemocytometer or plankton counting chamber, depending on cell size. The counts used for determining percent change in this parameter were averages of four replicates.

## RESULTS

*Cyclotella meneghiniana*

Detailed lipid class composition of *C. meneghiniana* for both control and exposed cultures is presented in Table 1. Percent change occurring in exposed cultures (compared with controls) for four parameters, cell count, chlorophyll *a*, chlorophyll *a*/neutral lipid ratio, and the neutral/polar lipid ratio are presented in Figure 1. At the time both exposures were initiated, (t=0), chlorophyll/neutral lipid ratios were relatively high and the neutral to polar lipid ratios were low (Table 1). On day 1, exposure to this isomer resulted in a reduction in chlorophyll in the 11th hour exposure and an increase in the neutral/polar lipid ratio. Increases in chlorophyll and the chlorophyll *a*/neutral lipid ratio were observed starting on day 3 and no further reductions were observed. Although cell counts were slightly diminished with exposure, by the 10th day, counts were virtually identical with control cultures. After the initial increase in neutral lipids, exposed cultures demonstrated a consistent decline in the neutral/polar lipid ratio, which appeared to result from increases in all polar lipid classes uniformly (Table 1).

*Melosira italica*

The overall neutral/polar lipid ratios of *M. italica* were the lowest of the four diatoms (Table 2). Values in the 8th hour of the light cycle are consistently lower than in the 11th hour. In general, *M. italica* appears to be more sensitive to this isomer than *C. meneghiniana* (Figure 2). Cell counts in cultures exposed in the 8th hour of the light cycle were reduced approximately 20% whereas those exposed in the 11th hour of the light period were reduced by ca. 50%. With the exception of chlorophyll measurements on days 1 and 10 in the 11th hour exposure which demonstrated high standard errors (Table 2), a general pattern of decreased chlorophyll *a*, decreased chlorophyll *a*/ neutral lipid ratio, and increased neutral/polar lipid ratio was observed in both exposures. However, it appears that effects in lipid class composition were more pronounced in the 8th hour exposure whereas cell counts effects were more pronounced in the 11th hour exposure.

*Melosira varians*

Lipid class composition of *M. varians* is presented in Table 3. In general, neutral/polar lipid ratios were consistent in the 8th and 11th hours of the light cycle in control cultures and these ratios declined with growth. Figure 3 demonstrates that cell counts were rather erratic for this taxon. However, reductions were observed in cell counts by day 10. Chlorophyll *a* as well as the chlorophyll *a*/ neutral lipid ratios were consistently reduced in both exposures. The greatest effect observed was the long term increase in the neutral/polar lipid ratio in the 8th hour exposure.

*Synedra filiformis*

Unlike the other three diatoms, *S. filiformis* demonstrated no short-term (i.e. 1 day) effect with exposure at either time (Figure 4). However, at the time exposures were initiated, the neutral/polar lipid ratio was lower at the 11th hour of the light period (Table 4). Cell counts were largely unaffected until the 10th day when an approximately 80 % reduction occurred in the 11th hour exposure. Chlorophyll and chlorophyll *a*/neutral lipid ratios increased in the 11th hour exposure between days 3 and 7. However, this ratio is reduced in both exposures by the 10th day. Although chlorophyll *a* appeared to be reduced with exposure to the chlorinated benzene, the neutral/polar lipid ratio decreased (Figure 4), largely as a result of increases in both AMPL and PL at 10 days. The magnitude of reduction was most similar to the data presented for *C. meneghiniana* (Figure 1).

**Table 1.** Lipid class composition of *Cyclotella meneghiniana*. Control and exposure cultures. Growth conditions 16:8 h L/D at 20°C. 2 pm samples were in the 8th hour of the light cycle; 5 pm samples were in the 11th hour of the light cycle. Selected ratios and % composition presented as % and standard error ( )

| Sample | HC | | WE/SE | | TG/FFA | | ALC | | ST | | Chl a | | Ampl | | PL | | Chl a/Neut | | Neut/Polar | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| days | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. |
| (A) = 8th hour | | | | | | | | | | | | | | | | | | | | |
| t = 0 | 3.7 | | 31.1 | | 28.7 | | 1.9 | | 4.2 | | 11.2 | | 4.4 | | 14.9 | | 0.17 | | 2.5 | |
| | (0.3) | | (3.9) | | (3.9) | | (0.6) | | (0.4) | | (0.9) | | (0.6) | | (2.8) | | (0.02) | | (0.4) | |
| t = 1 | 3.6 | 3.4 | 26.6 | 24.0 | 25.2 | 40.5 | 2.3 | 0.5 | 5.6 | 4.1 | 9.5 | 9.4 | 6.3 | 4.5 | 20.8 | 13.5 | 0.15 | 0.13 | 1.7 | 2.7 |
| | (0.9) | (0.3) | (1.4) | (1.1) | (0.2) | (2.6) | (0.4) | (0.5) | (0.7) | (0.1) | (2.3) | (0.3) | (0.6) | (0.8) | (2.0) | (1.3) | (0.04) | (0.01) | (0.1) | (0.3) |
| t = 3 | 5.9 | 3.6 | 29.4 | 33.4 | 29.6 | 30.9 | 1.4 | 1.6 | 3.9 | 3.8 | 5.2 | 9.1 | 7.9 | 4.1 | 16.6 | 13.4 | 0.07 | 0.12 | 2.5 | 2.8 |
| | (1.0) | (0.9) | (3.0) | (1.5) | (1.3) | (2.1) | (0.5) | (0.6) | (0.2) | (1.0) | (1.5) | (2.5) | (1.4) | (1.3) | (3.6) | (2.8) | (0.02) | (0.03) | (0.4) | (0.3) |
| t - 5 | 3.1 | 4.2 | 24.1 | 17.2 | 35.1 | 26.3 | 1.2 | 2.3 | 7.5 | 8.1 | 7.3 | 23.7 | 6.6 | 1.6 | 15.2 | 16.6 | 0.10 | 0.42 | 2.5 | 1.4 |
| | (0.1) | (0.4) | (1.2) | (1.0) | (1.9) | (2.1) | (0.4) | (1.4) | (1.3) | (0.6) | (0.6) | (1.4) | (0.5) | (0.5) | (1.6) | (1.7) | (0.01) | (0.05) | (0.2) | (0.2) |
| t = 7 | 4.2 | 3.4 | 12.9 | 15.4 | 46.0 | 31.4 | 0.7 | 0.0 | 6.5 | 4.1 | 13.0 | 20.3 | 5.0 | 6.1 | 11.7 | 19.1 | 0.19 | 0.38 | 2.5 | 1.2 |
| | (1.3) | (0.5) | (2.2) | (3.5) | (1.7) | (2.7) | (0.4) | (0.0) | (1.1) | (0.2) | (2.9) | (2.5) | (0.9) | (0.8) | (1.1) | (0.9) | (0.05) | (0.05) | (0.4) | (0.1) |
| t = 10 | 3.5 | 3.0 | 40.4 | 24.9 | 23.9 | 32.0 | 2.3 | 1.5 | 4.0 | 5.8 | 6.4 | 8.5 | 7.4 | 6.0 | 11.9 | 18.3 | 0.09 | 0.13 | 3.2 | 2.2 |
| | (0.5) | (0.1) | (11.8) | (9.3) | (8.3) | (5.0) | (1.1) | (0.8) | (0.4) | (2.2) | (2.2) | (0.6) | (3.5) | (1.5) | (3.8) | (2.5) | (0.03) | (0.01) | (0.8) | (0.4) |

(continued)

**Table 1.** (continued)

| Sample | HC | | WE/SE | | TG/FFA | | ALC | | ST | | Chl *a* | | Ampl | | PL | | Chl *a*/Neut | | Neut/Polar | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| days | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. |
| (B) = 11th hour | | | | | | | | | | | | | | | | | | | | |
| t = 0 | 3.3 | | 18.0 | | 47.5 | | 1.4 | | 5.8 | | 10.6 | | 3.2 | | 10.2 | | 0.15 | | 3.2 | |
| | (0.6) | | (3.9) | | (4.7) | | (0.4) | | (0.7) | | (1.3) | | (0.3) | | (1.5) | | (0.02) | | (0.2) | |
| t = 1 | 3.3 | 2.5 | 23.6 | 37.5 | 39.1 | 28.7 | 2.4 | 2.3 | 4.2 | 3.7 | 9.5 | 3.6 | 3.9 | 3.8 | 14.0 | 18.0 | 0.14 | 0.05 | 2.8 | 3.7 |
| | (0.1) | (0.2) | (2.7) | (5.6) | (1.5) | (1.5) | (0.2) | (0.3) | (0.4) | (0.3) | (1.0) | (0.1) | (1.4) | (0.7) | (2.3) | (6.5) | (0.01) | (0.01) | (0.5) | (1.0) |
| t = 3 | 3.3 | 3.6 | 21.6 | 25.7 | 40.1 | 34.3 | 1.3 | 0.0 | 6.1 | 4.2 | 12.8 | 12.1 | 3.6 | 5.0 | 11.2 | 15.1 | 0.20 | 0.18 | 2.7 | 2.2 |
| | (0.4) | (0.4) | (2.9) | (1.3) | (0.7) | (3.0) | (0.6) | (0.0) | (1.5) | (0.7) | (0.6) | (1.8) | (0.5) | (1.3) | (0.7) | (3.4) | (0.02) | (0.02) | (0.2) | (0.3) |
| t = 5 | 2.9 | 3.4 | 34.6 | 28.4 | 24.3 | 24.3 | 2.0 | 0.6 | 8.9 | 9.5 | 9.2 | 14.8 | 4.3 | 5.9 | 13.8 | 13.0 | 0.15 | 0.24 | 2.8 | 2.2 |
| | (0.7) | (0.4) | (7.2) | (8.1) | (6.2) | (1.1) | (0.7) | (0.6) | (3.3) | (2.5) | (2.7) | (3.9) | (0.7) | (1.2) | (0.9) | (2.0) | (0.05) | (0.08) | (0.3) | (0.5) |
| t = 7 | 1.6 | 2.6 | 39.2 | 27.9 | 33.7 | 34.3 | 0.8 | 1.6 | 4.0 | 5.0 | 8.2 | 10.5 | 3.6 | 5.2 | 8.9 | 12.8 | 0.11 | 0.16 | 4.1 | 2.6 |
| | (0.6) | (0.6) | (11.7) | (1.5) | (8.8) | (1.7) | (0.5) | (1.1) | (0.8) | (0.8) | (2.0) | (2.1) | (0.5) | (0.9) | (1.0) | (1.4) | (0.03) | (0.04) | (0.6) | (0.4) |
| t = 10 | 2.6 | 6.0 | 34.4 | 32.0 | 33.5 | 23.2 | 2.1 | 2.2 | 2.7 | 4.9 | 4.7 | 8.1 | 6.5 | 5.9 | 13.6 | 17.7 | 0.06 | 0.13 | 3.1 | 2.2 |
| | (1.0) | (0.1) | (9.0) | (1.3) | (7.6) | (0.3) | (0.8) | (0.0) | (1.0) | (0.1) | (1.3) | (1.6) | (1.5) | (0.8) | (1.1) | (2.5) | (0.02) | (0.02) | (0.3) | (0.2) |

**Table 2.** Lipid class composition of *Melosira italica*. Control and exposure cultures. Growth conditions 16:8 h L/D at 20°C. 2 pm samples were in the 8th hour hour of the light cycle; 5 pm samples were in the 11th hour of the light cycle. Selected ratios and % composition presented as % and standard error ( )

| Sample | HC | | WE/SE | | TG/FFA | | ALC | | ST | | Chl *a* | | Ampl | | PL | | Chl a/Neut | | Neut/Polar | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| days | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. |
| (A) = 8th hour | | | | | | | | | | | | | | | | | | | | |
| t = 0 | 1.4 | | 18.9 | | 27.0 | | 2.6 | | 9.5 | | 5.1 | | 11.9 | | 23.7 | | 0.08 | | 1.7 | |
| | (0.1) | | (3.9) | | (2.4) | | (0.7) | | (0.7) | | (1.6) | | (3.6) | | (2.7) | | (0.03) | | (0.3) | |
| t = 1 | 2.3 | 1.3 | 17.8 | 23.1 | 34.0 | 37.2 | 0.8 | 3.1 | 6.0 | 7.9 | 13.6 | 7.7 | 3.6 | 6.0 | 21.7 | 13.8 | 0.26 | 0.11 | 1.9 | 2.7 |
| | (0.5) | (0.1) | (1.6) | (1.6) | (7.9) | (2.3) | (0.5) | (0.1) | (0.5) | (1.8) | (4.8) | (3.9) | (1.9) | (2.3) | (1.8) | (1.9) | (0.12) | (0.06) | (0.5) | (0.3) |
| t = 3 | 2.2 | 2.1 | 10.6 | 17.5 | 36.1 | 25.2 | 2.4 | 4.0 | 9.5 | 8.3 | 11.6 | 7.3 | 13.2 | 8.4 | 14.3 | 27.1 | 0.18 | 0.12 | 1.6 | 1.4 |
| | (0.4) | (0.2) | (4.0) | (1.9) | (5.2) | (4.6) | (1.3) | (0.3) | (1.1) | (1.4) | (6.5) | (3.8) | (5.5) | (3.7) | (3.2) | (4.8) | (0.10) | (0.06) | (0.2) | (0.2) |
| t = 5 | 2.1 | 2.3 | 10.5 | 27.0 | 29.6 | 30.6 | 1.0 | 3.9 | 8.7 | 5.9 | 15.5 | 0.4 | 16.7 | 7.5 | 15.8 | 22.3 | 0.28 | 0.01 | 1.1 | 2.3 |
| | (0.3) | (0.2) | (1.4) | (5.3) | (3.2) | (3.2) | (1.0) | (0.5) | (0.9) | (1.5) | (5.1) | (0.0) | (10.7) | (1.5) | (4.2) | (1.3) | (0.09) | (0.00) | (0.2) | (0.2) |
| t = 7 | 2.7 | 2.1 | 10.4 | 9.1 | 42.3 | 50.3 | 1.1 | 3.3 | 10.2 | 9.4 | 0.9 | 1.2 | 13.9 | 9.4 | 18.5 | 15.3 | 0.01 | 0.02 | 2.3 | 2.9 |
| | (0.4) | (0.2) | (1.3) | (1.8) | (4.1) | (1.7) | (0.7) | (0.6) | (0.3) | (0.6) | (0.8) | (1.1) | (3.3) | (2.8) | (3.2) | (2.2) | (0.01) | (0.01) | (0.6) | (0.3) |
| t = 10 | 3.0 | 1.8 | 20.9 | 19.2 | 22.4 | 44.1 | 2.4 | 3.1 | 6.6 | 10.1 | 22.0 | 7.3 | 2.6 | 4.5 | 20.2 | 10.0 | 0.44 | 0.10 | 1.4 | 4.3 |
| | (0.4) | (0.4) | (2.8) | (2.8) | (1.7) | (6.2) | (1.3) | (0.2) | (2.0) | (1.0) | (3.4) | (2.2) | (1.0) | (1.1) | (3.9) | (3.5) | (0.12) | (0.03) | (0.4) | (1.1) |

(continued)

**Table 2.** (continued)

| Sample | HC | | WE/SE | | TG/FFA | | ALC | | ST | | Chl *a* | | Ampl | | PL | | Chl *a*/Neut | | Neut/Polar | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| days | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. |
| (B) = 11th hour | | | | | | | | | | | | | | | | | | | | |
| t = 0 | 2.2 | | 15.6 | | 36.8 | | 4.1 | | 10.5 | | 13.1 | | 5.9 | | 11.8 | | 0.19 | | 2.3 | |
| | (0.2) | | (1.7) | | (1.6) | | (1.1) | | (0.9) | | (3.1) | | (2.4) | | (1.2) | | (0.06) | | (0.2) | |
| t = 1 | 2.2 | 3.0 | 25.6 | 14.1 | 26.6 | 28.7 | 3.8 | 2.1 | 6.5 | 9.0 | 2.0 | 6.7 | 12.9 | 8.5 | 20.5 | 28.1 | 0.03 | 0.12 | 1.8 | 1.3 |
| | (0.5) | (0.3) | (1.1) | (2.7) | (1.3) | (1.5) | (0.3) | (0.7) | (0.9) | (0.7) | (1.0) | (2.7) | (3.3) | (3.4) | (1.0) | (1.2) | (0.01) | (0.05) | (0.1) | (0.2) |
| t = 3 | 1.8 | 2.5 | 13.9 | 15.4 | 36.4 | 49.5 | 2.4 | 2.6 | 10.3 | 7.2 | 9.1 | 3.8 | 7.4 | 6.4 | 18.6 | 12.6 | 0.13 | 0.05 | 2.0 | 3.4 |
| | (0.1) | (0.2) | (2.9) | (4.3) | (4.0) | (2.6) | (0.9) | (0.6) | (0.7) | (0.7) | (3.2) | (1.2) | (4.6) | (2.2) | (3.8) | (1.7) | (0.05) | (0.02) | (0.4) | (0.3) |
| t = 5 | 2.6 | 1.7 | 21.6 | 24.3 | 35.1 | 38.4 | 1.0 | 1.4 | 7.6 | 9.0 | 5.4 | 2.1 | 8.4 | 8.1 | 18.3 | 15.0 | 0.08 | 0.03 | 2.6 | 3.3 |
| | (0.5) | (0.2) | (4.6) | (3.8) | (10.1) | (6.3) | (0.6) | (0.8) | (1.2) | (1.2) | (2.1) | (1.3) | (5.0) | (2.4) | (3.4) | (2.9) | (0.03) | (0.02) | (0.7) | (0.8) |
| t = 7 | 3.2 | 2.4 | 12.8 | 24.0 | 29.5 | 30.8 | 3.4 | 3.5 | 9.2 | 7.8 | 14.9 | 6.5 | 4.9 | 4.5 | 22.1 | 20.4 | 0.28 | 0.10 | 1.4 | 2.2 |
| | (0.7) | (0.3) | (3.1) | (0.9) | (3.3) | (0.8) | (1.3) | (0.6) | (2.1) | (0.2) | (7.9) | (0.8) | (3.0) | (1.6) | (2.1) | (2.3) | (0.16) | (0.01) | (0.2) | (0.2) |
| t = 10 | 2.6 | 1.5 | 18.8 | 14.5 | 43.8 | 35.7 | 3.4 | 4.4 | 9.2 | 12.9 | 9.4 | 16.5 | 5.3 | 1.9 | 7.5 | 12.4 | 0.12 | 0.27 | 3.5 | 2.6 |
| | (0.3) | (0.1) | (1.9) | (0.6) | (2.5) | (7.5) | (0.7) | (0.5) | (0.6) | (1.3) | (4.2) | (6.8) | (1.9) | (1.0) | (2.7) | (0.7) | (0.05) | (0.13) | (0.2) | (0.7) |

**Table 3.** Lipid class composition of *Melosira varians*. Control and exposure cultures. Growth conditions 16:8 h L/D at 20°C. 2 pm samples were in the 8th hour of the light cycle; 5 pm samples were in the 11th hour of the light cycle. Selected ratios and % composition presented as % and standard error ( )

| Sample | HC | | WE/SE | | TG/FFA | | ALC | | ST | | Chl a | | Ampl | | PL | | Chl a/Neut | | Neut/Polar | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| days | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. |
| (A) = 8th hour | | | | | | | | | | | | | | | | | | | | |
| t = 0 | 2.6 | | 13.9 | | 49.7 | | 2.7 | | 5.8 | | 5.1 | | 6.1 | | 14.0 | | 0.07 | | 3.0 | |
| | (0.3) | | (0.7) | | (1.0) | | (0.6) | | (0.9) | | (0.6) | | (0.6) | | (0.5) | | (0.01) | | (0.1) | |
| t = 1 | 2.5 | 2.0 | 26.1 | 12.9 | 33.5 | 45.5 | 3.2 | 1.5 | 7.6 | 6.1 | 3.8 | 7.8 | 8.3 | 7.6 | 14.9 | 16.5 | 0.05 | 0.12 | 3.4 | 2.6 |
| | (0.3) | (0.4) | (2.2) | (1.8) | (4.7) | (4.0) | (0.4) | (0.9) | (0.2) | (0.7) | (1.6) | (1.2) | (2.4) | (0.7) | (5.9) | (4.7) | (0.03) | (0.03) | (1.0) | (0.9) |
| t = 3 | 2.8 | 2.6 | 21.5 | 27.9 | 28.5 | 22.4 | 2.2 | 3.4 | 6.7 | 6.0 | 11.2 | 3.5 | 3.6 | 12.1 | 23.5 | 22.1 | 0.20 | 0.06 | 1.8 | 1.8 |
| | (0.3) | (0.4) | (3.0) | (2.5) | (4.4) | (2.4) | (1.1) | (0.5) | (1.9) | (0.8) | (3.0) | (1.1) | (0.9) | (3.5) | (3.2) | (1.8) | (0.07) | (0.01) | (0.4) | (0.4) |
| t = 5 | 2.8 | 2.3 | 27.0 | 30.8 | 30.4 | 29.3 | 3.1 | 2.9 | 6.8 | 5.6 | 5.6 | 3.6 | 5.5 | 10.9 | 18.8 | 14.5 | 0.08 | 0.05 | 2.5 | 2.6 |
| | (0.3) | (0.2) | (2.0) | (2.3) | (4.9) | (2.8) | (0.4) | (0.4) | (0.5) | (0.7) | (0.7) | (0.8) | (1.2) | (3.2) | (3.4) | (1.4) | (0.01) | (0.01) | (0.5) | (0.4) |
| t = 7 | 3.4 | 3.1 | 17.7 | 23.9 | 34.6 | 41.9 | 2.2 | 3.2 | 8.5 | 6.2 | 7.7 | 6.3 | 5.4 | 5.0 | 20.5 | 10.1 | 0.12 | 0.08 | 2.2 | 4.0 |
| | (0.5) | (0.5) | (5.4) | (2.4) | (8.4) | (4.3) | (0.8) | (0.5) | (0.6) | (0.9) | (1.3) | (1.4) | (0.2) | (0.8) | (3.1) | (2.5) | (0.03) | (0.02) | (0.5) | (0.8) |
| t = 10 | 1.8 | 6.8 | 24.9 | 34.2 | 27.6 | 32.4 | 2.8 | 4.3 | 7.9 | 5.3 | 6.8 | 5.3 | 7.7 | 6.1 | 20.4 | 5.6 | 0.11 | 0.06 | 1.9 | 5.1 |
| | (0.1) | (0.2) | (1.1) | (0.3) | (2.0) | (1.9) | (1.1) | (0.1) | (1.8) | (0.2) | (1.0) | (0.4) | (1.4) | (1.2) | (0.8) | (0.6) | (0.02) | (0.01) | (0.2) | (0.8) |

(continued)

Table 3. (continued)

| Sample | HC | | WE/SE | | TG/FFA | | ALC | | ST | | Chl a | | Ampl | | PL | | Chl a/Neut | | Neut/Polar | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| days | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. |
| (B) = 11th hour | | | | | | | | | | | | | | | | | | | | |
| t = 0 | 2.4 | | 12.1 | | 48.6 | | 1.3 | | 7.5 | | 5.7 | | 7.0 | | 15.5 | | 0.08 | | 2.9 | |
| | (0.2) | | (2.6) | | (6.2) | | (0.5) | | (0.4) | | (0.7) | | (1.0) | | (2.5) | | (0.01) | | (0.4) | |
| t = 1 | 3.3 | 2.2 | 16.6 | 21.4 | 38.3 | 33.7 | 3.6 | 3.1 | 8.8 | 6.3 | 7.0 | 5.6 | 5.0 | 7.8 | 17.4 | 19.8 | 0.10 | 0.09 | 2.5 | 2.1 |
| | (0.6) | (0.4) | (1.8) | (5.0) | (1.2) | (1.1) | (0.3) | (0.5) | (0.5) | (0.4) | (1.1) | (0.8) | (0.6) | (0.8) | (2.5) | (3.1) | (0.02) | (0.02) | (0.2) | (0.3) |
| t = 3 | 2.9 | 3.1 | 12.1 | 28.0 | 40.1 | 26.6 | 1.2 | 3.1 | 11.1 | 5.2 | 5.8 | 4.7 | 6.8 | 7.4 | 20.0 | 21.9 | 0.09 | 0.07 | 2.1 | 2.0 |
| | (0.1) | (0.1) | (0.2) | (2.3) | (0.3) | (1.5) | (0.8) | (0.2) | (0.1) | (0.3) | (2.8) | (0.9) | (1.3) | (1.5) | (0.2) | (1.7) | (0.04) | (0.01) | (0.1) | (0.3) |
| t = 5 | 3.6 | 3.6 | 13.7 | 18.1 | 24.8 | 25.5 | 1.2 | 3.1 | 8.4 | 7.6 | 10.4 | 7.2 | 8.3 | 7.9 | 29.6 | 27.0 | 0.20 | 0.13 | 1.2 | 1.4 |
| | (0.7) | (0.7) | (0.6) | (3.8) | (4.8) | (1.5) | (1.1) | (0.2) | (0.3) | (0.9) | (3.5) | (2.0) | (3.4) | (2.2) | (5.2) | (1.5) | (0.05) | (0.04) | (0.2) | (0.2) |
| t = 7 | 2.6 | 3.4 | 16.1 | 20.6 | 28.9 | 27.9 | 3.2 | 1.9 | 5.9 | 6.0 | 8.2 | 3.1 | 9.6 | 14.8 | 25.5 | 22.3 | 0.16 | 0.05 | 1.5 | 1.5 |
| | (0.6) | (0.5) | (1.4) | (2.2) | (3.7) | (2.3) | (0.3) | (0.7) | (0.3) | (0.7) | (2.1) | (1.2) | (0.9) | (1.8) | (3.2) | (2.2) | (0.05) | (0.02) | (0.4) | (0.1) |
| t = 10 | 2.9 | 3.9 | 22.7 | 16.1 | 38.5 | 46.4 | 2.7 | 2.3 | 4.4 | 5.0 | 2.8 | 2.9 | 9.3 | 7.5 | 16.8 | 15.8 | 0.04 | 0.04 | 2.6 | 2.9 |
| | (0.1) | (0.3) | (1.3) | (0.9) | (2.6) | (3.1) | (0.4) | (1.0) | (0.7) | (0.3) | (1.1) | (1.0) | (1.0) | (1.9) | (3.9) | (2.2) | (0.01) | (0.01) | (0.4) | (0.3) |

**Table 4.** Lipid class composition of *Synedra filiformis*. Control and exposure cultures. Growth conditions 16:8 h L/D at 20°C. 2 pm samples were in the 8th hour of the light cycle; 5 pm samples were in the 11th hour of the light cycle. Selected ratios and % composition presented as % and standard error ( )

| Sample | HC | | WE/SE | | TG/FFA | | ALC | | ST | | Chl a | | Ampl | | PL | | Chl a/Neut | | Neut/Polar | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| days | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. |
| (A) = 8th hour | | | | | | | | | | | | | | | | | | | | |
| t = 0 | 2.0 | | 17.4 | | 38.7 | | 2.5 | | 10.5 | | 9.5 | | 3.4 | | 16.1 | | 0.14 | | 2.5 | |
| | (0.3) | | (3.2) | | (3.6) | | (0.5) | | (0.6) | | (0.8) | | (0.7) | | (1.6) | | (0.01) | | (0.2) | |
| t = 1 | 2.6 | 1.9 | 16.0 | 14.2 | 35.0 | 39.2 | 1.8 | 2.6 | 9.6 | 11.4 | 10.0 | 10.6 | 4.4 | 4.2 | 20.7 | 15.9 | 0.16 | 0.15 | 1.9 | 2.4 |
| | (0.3) | (0.2) | (1.9) | (2.2) | (2.2) | (1.7) | (0.6) | (0.3) | (0.7) | (0.6) | (0.4) | (0.9) | (0.9) | (1.1) | (2.4) | (1.8) | (0.01) | (0.02) | (0.3) | (0.3) |
| t = 3 | 3.0 | 2.3 | 13.9 | 13.9 | 28.8 | 31.5 | 2.1 | 2.3 | 13.0 | 13.5 | 13.0 | 10.6 | 4.3 | 3.4 | 22.1 | 22.5 | 0.21 | 0.17 | 1.6 | 1.8 |
| | (0.7) | (0.2) | (2.1) | (2.2) | (1.4) | (3.3) | (0.7) | (0.4) | (0.8) | (1.3) | (2.0) | (1.5) | (0.8) | (1.2) | (4.2) | (2.5) | (0.03) | (0.02) | (0.2) | (0.2) |
| t = 5 | 2.7 | 2.5 | 14.0 | 16.5 | 36.8 | 33.3 | 2.7 | 2.3 | 13.8 | 12.2 | 11.3 | 11.2 | 5.5 | 5.5 | 13.4 | 16.5 | 0.17 | 0.17 | 2.8 | 2.1 |
| | (0.3) | (0.3) | (3.7) | (1.8) | (3.7) | (4.1) | (1.0) | (0.9) | (0.5) | (0.5) | (1.0) | (1.8) | (1.7) | (2.6) | (5.0) | (1.2) | (0.03) | (0.03) | (0.8) | (0.3) |
| t = 7 | 2.6 | 2.3 | 12.1 | 18.9 | 38.0 | 25.9 | 3.1 | 0.8 | 13.8 | 11.9 | 12.4 | 12.3 | 3.6 | 6.2 | 14.5 | 21.8 | 0.18 | 0.21 | 2.3 | 1.5 |
| | (0.2) | (0.1) | (3.7) | (2.8) | (3.1) | (3.0) | (0.5) | (0.8) | (0.8) | (1.2) | (1.5) | (2.4) | (1.0) | (2.0) | (1.5) | (2.3) | (0.02) | (0.04) | (0.2) | (0.1) |
| t = 10 | 2.8 | 2.5 | 15.7 | 25.4 | 35.6 | 31.2 | 2.0 | 1.2 | 11.0 | 10.9 | 10.6 | 8.1 | 5.1 | 3.8 | 17.4 | 16.9 | 0.16 | 0.11 | 2.2 | 2.5 |
| | (0.2) | (0.2) | (2.3) | (0.5) | (2.0) | (1.0) | (0.8) | (0.7) | (0.8) | (0.7) | (2.1) | (1.0) | (1.8) | (0.6) | (2.6) | (0.7) | (0.04) | (0.01) | (0.5) | (0.0) |

continued)

**Table 4.** *(continued)*

| Sample | HC | | WE/SE | | TG/FFA | | ALC | | ST | | Chl *a* | | Ampl | | PL | | Chl *a*/Neut | | Neut/Polar | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| days | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. | Cont. | Exp. |
| (B) = 11th hour | | | | | | | | | | | | | | | | | | | | |
| t = 0 | 2.1 | | 14.1 | | 35.9 | | 1.3 | | 10.2 | | 10.3 | | 5.5 | | 20.6 | | 0.16 | | 2.0 | |
| | (0.3) | | (2.3) | | (3.8) | | (0.3) | | (0.7) | | (0.7) | | (0.9) | | (4.3) | | (0.01) | | (0.4) | |
| t = 1 | 2.1 | 1.9 | 18.1 | 13.0 | 33.2 | 42.1 | 2.7 | 1.9 | 8.7 | 9.4 | 9.5 | 9.2 | 4.1 | 3.6 | 21.8 | 19.0 | 0.15 | 0.14 | 1.9 | 2.2 |
| | (0.3) | (0.2) | (2.2) | (1.5) | (2.8) | (3.5) | (0.1) | (0.3) | (0.8) | (0.4) | (0.9) | (0.7) | (1.2) | (1.1) | (3.1) | (1.6) | (0.02) | (0.01) | (0.3) | (0.3) |
| t = 3 | 2.4 | 3.3 | 19.6 | 14.0 | 36.0 | 37.8 | 3.1 | 2.3 | 11.1 | 11.4 | 7.8 | 13.5 | 5.3 | 4.4 | 14.8 | 13.3 | 0.11 | 0.21 | 3.6 | 2.5 |
| | (0.2) | (0.3) | (1.4) | (3.7) | (7.0) | (1.4) | (0.4) | (0.8) | (1.2) | (0.6) | (1.6) | (3.1) | (1.4) | (1.4) | (7.9) | (3.4) | (0.03) | (0.06) | (1.3) | (0.6) |
| t = 5 | 1.5 | 2.4 | 13.8 | 7.7 | 22.9 | 29.7 | 1.7 | 1.2 | 14.4 | 13.3 | 10.6 | 16.2 | 7.5 | 3.4 | 27.7 | 26.2 | 0.20 | 0.31 | 1.3 | 1.2 |
| | (0.6) | (0.3) | (2.8) | (1.4) | (2.8) | (2.2) | (0.5) | (0.7) | (0.8) | (0.8) | (1.4) | (1.5) | (1.2) | (0.7) | (3.4) | (2.5) | (0.03) | (0.04) | (0.3) | (0.2) |
| t = 7 | 2.2 | 2.9 | 22.3 | 13.9 | 35.9 | 45.0 | 2.4 | 0.7 | 11.3 | 11.2 | 7.6 | 8.6 | 5.3 | 4.5 | 13.0 | 13.2 | 0.11 | 0.12 | 4.2 | 3.5 |
| | (0.3) | (0.2) | (3.8) | (2.0) | (3.0) | (8.8) | (0.6) | (0.7) | (0.6) | (0.2) | (1.1) | (0.9) | (1.0) | (0.4) | (6.2) | (6.6) | (0.03) | (0.01) | (1.6) | (1.1) |
| t = 10 | 3.3 | 3.0 | 14.7 | 18.2 | 34.3 | 23.8 | 2.7 | 1.3 | 13.5 | 4.7 | 8.4 | 5.1 | 6.6 | 11.5 | 16.5 | 32.4 | 0.13 | 0.09 | 2.3 | 1.1 |
| | (0.2) | (0.3) | (1.0) | (2.2) | (2.0) | (2.2) | (0.3) | (0.7) | (0.9) | (1.9) | (0.8) | (2.8) | (1.1) | (4.3) | (3.6) | (4.0) | (0.02) | (0.05) | (0.3) | (0.2) |

*Total Extractable Lipid (TEL) as the Time of Exposure*

TEL as a percent of dry weight for cultures at the onset of exposure are presented in Table 5. Values reported are considerably lower than those presented in our previous paper. However, trends are similar for *C. meneghiniana* and *M. varians*. For *Cyclotella,* TEL appears to be constant, although perhaps slightly lower in the 8th hour of the light period. Significant increases in TEL are observed in the 8th hour of light in *M. varians* and in the 11th hour of light in *S. filiformis*.

**Table 5.** Total extractable lipid (TEL) of cultures at onset of exposure. Values reported are percent of dry weight ± (standard error.)

| Organism | 8th hr | 11th hr |
|---|---|---|
| *Cyclotella meneghiniana* | 5.4 (0.6) | 5.7 (1.5) |
| *Melosira italica* | 5.3 (0.8) | 5.1 (1.0) |
| *Melosira varians* | 9.4 (1.4) | 6.9 (0.7) |
| *Synedra filiformis* | 6.1 (1.0) | 8.0 (1.3) |

## DISCUSSION

Our previous short-term experiments with *Cyclotella* (Sicko-Goad *et al.* 1989a-d; Sicko-Goad and Andresen 1992) and *M. varians* (Sicko-Goad and Andresen 1992) indicated *M. varians* was more sensitive to exposure to 1,3,5-trichlorobenzene. The data presented here suggest both species of *Melosira* are more sensitive to 1,2,3-trichlorobenzene than *Cyclotella.* Furthermore, in the experiment conducted longer than 5 days, no reductions in cell count are observed in *Cyclotella,* and chlorophyll *a* content is actually higher in cells exposed to this isomer. In contrast, both *M. varians* and *M. italica* are sensitive to long-term exposure to this isomer. Reductions in cell counts as well as reductions in the chlorophyll *a*/neutral lipid ratio are observed. The neutral/polar lipid ratio increases in both species, with a more pronounced time effect when the exposure is initiated in the 8th hour of the light period.

*Synedra filiformis* is largely unaffected by the isomer from the lipid perspective. Cell counts vary little with exposure and the neutral/polar lipid ratio actually declines. However, a decline in cell count is observed at 10 days, when exposure is initiated in the 11th hour of the light period.

The short-term effects (i.e. 1-3 day) observed with both 1,3,5,- and 1,2,3-trichlorobenzene are similar and appear to be related to the low triacylglycerol content and high polar lipid content in both the 8th and 11th hour exposures in *Cyclotella.* Of the two *Melosira* species, *M. italica* experienced more overall effects than *M. varians*. Of the four organisms studied, *M. italica* had the lowest neutral/polar lipid ratios, suggesting that toxicity effects are much more pronounced in rapidly growing cells, which is consistent with our previous observations. Where small declines were observed in cell numbers (11th hour), the neutral/polar lipid ratio was lower when the exposure was initiated.

Longer term reductions in cell counts were observed in *M. varians* when exposed in the 8th hour of the light cycle and in *S. filiformis* when exposed in the 11th hour of the light period. Since TEL

was higher in these cultures at the time of exposure, we consider this further evidence that increased lipid content at the time of exposure may lead to longer term effects.

During the course of the experiments some macroscopic changes were observed in two taxa. For *Melosira varians* cultures, the normal growth condition is for filaments to form loosely adherent, hair-like clumps. In exposed flasks the hair-like masses began to break up or decrease in size by the third day, and by the fifth day, no clumps were observed. This effect was most pronounced in the eighth hour exposure. Mucilage secretion, which facilitates clumping, apparently had been affected by the toxicant. *Synedra filiformis* is normally planktonic, however, in culture it adopts the growth habit of loosely attaching itself to a substrate by means of a mucilage pad, as a bushy, stellate colony in the manner of other members of the genus. Control cultures demonstrated this habit. Both exposed cultures did not exhibit the attaching growth habit but rather became single-celled. Thus in two unrelated taxa mucilage secretion was affected suggesting other metabolic systems are disrupted by exposure to chlorinated benzenes.

It has been suggested that sensitivity of algae to toxicants varies with season of the year, nutrient conditions, and physiological state of the cell (Hannan and Patouillet 1972; Conter *et al.* 1987; Neumann *et al.* 1987; Winner and Owen 1991). Our results further suggest that even under conditions of rapid growth, diel variations in physiological parameters are sufficient to result in different effects with a lipophilic toxicant is introduced at different times of the day. This is not surprising in view of the increasing reports on the variety of physiological parameters which undergo diel fluctuations (Sournia 1974; Varnum *et al.* 1986; Villareal and Carpenter 1990; Sundberg and Nilshammer-Holmvall 1975; Harding *et al.* 1983; Erga and Skjoldal 1990; Pettersson and Sahlsten 1990; Granata 1991; Sukenik and Carmeli 1990).

The diatoms utilized in this study were chosen to represent a variety of environmental trophic conditions. *Melosira varians* and *C. meneghiniana* have worldwide distributions and may be characterized as taxa of eutrophic waters (Hustedt 1938-1939; Stoermer and Ladewski 1976). *Melosira italica* and *Synedra filiformis* may be classified as oligotrophic to mesotrophic species (Stoermer and Ladewski 1976). The responses observed here do not appear to be correlated with observed distribution patterns in nature. That is, the cleaner water forms were not necessarily more susceptible to toxicant exposure. The most obvious correlations were with lipid content and composition.

In summary, it appears that lipid content and composition play an important role in predicting both short and long-term effects of lipophilic toxicants. Exposures initiated during periods of high polar lipid content, or when polar lipids are increasing usually result in immediate effects. Exposures commencing during periods of high neutral lipid content, high TEL content, or periods when neutral lipids are increasing most often result in delayed effects. Lipid content and composition vary through a diel cycle. Consequently, exposures initiated at different times of the day may produce quite different results.

## ACKNOWLEDGMENTS

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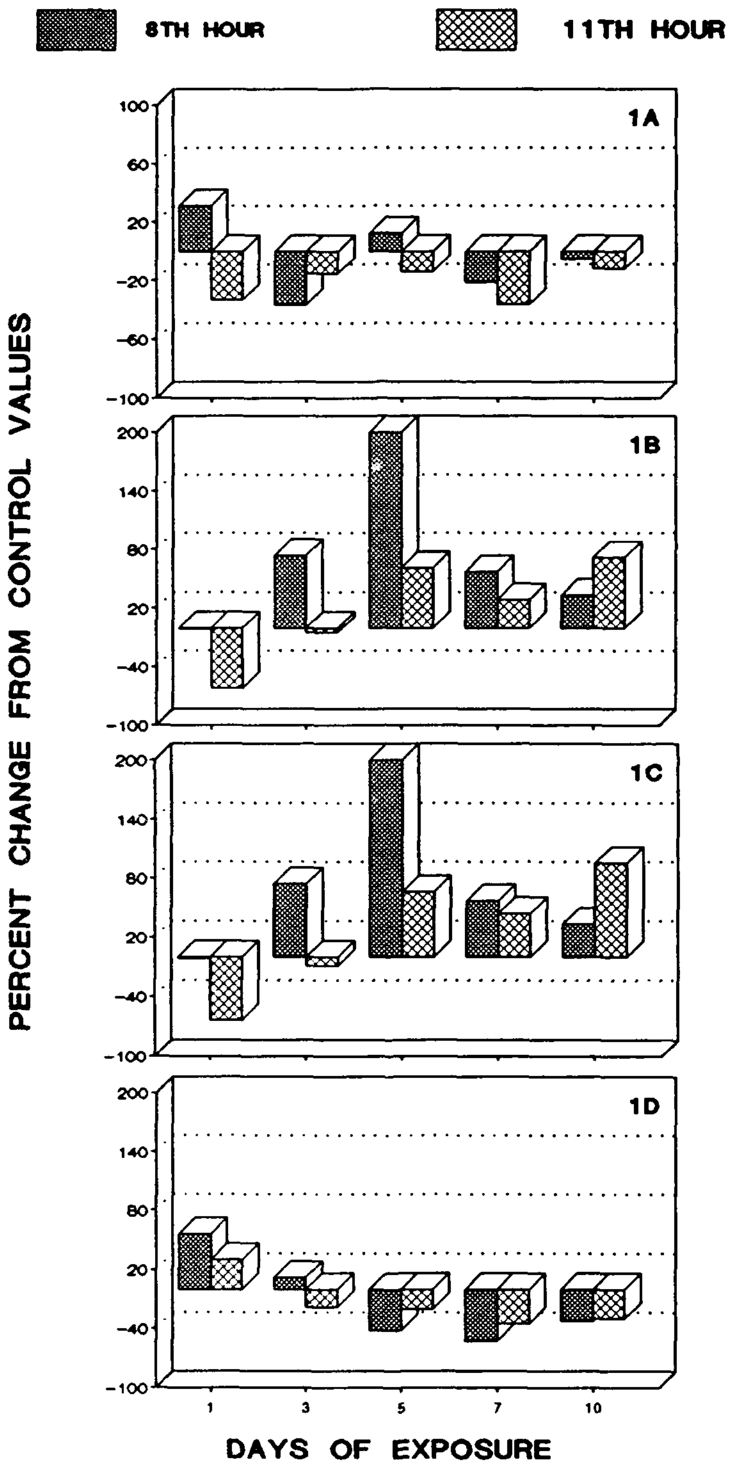
## LIST OF FIGURES



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PERCENT CHANGE FROM CONTROL VALUES
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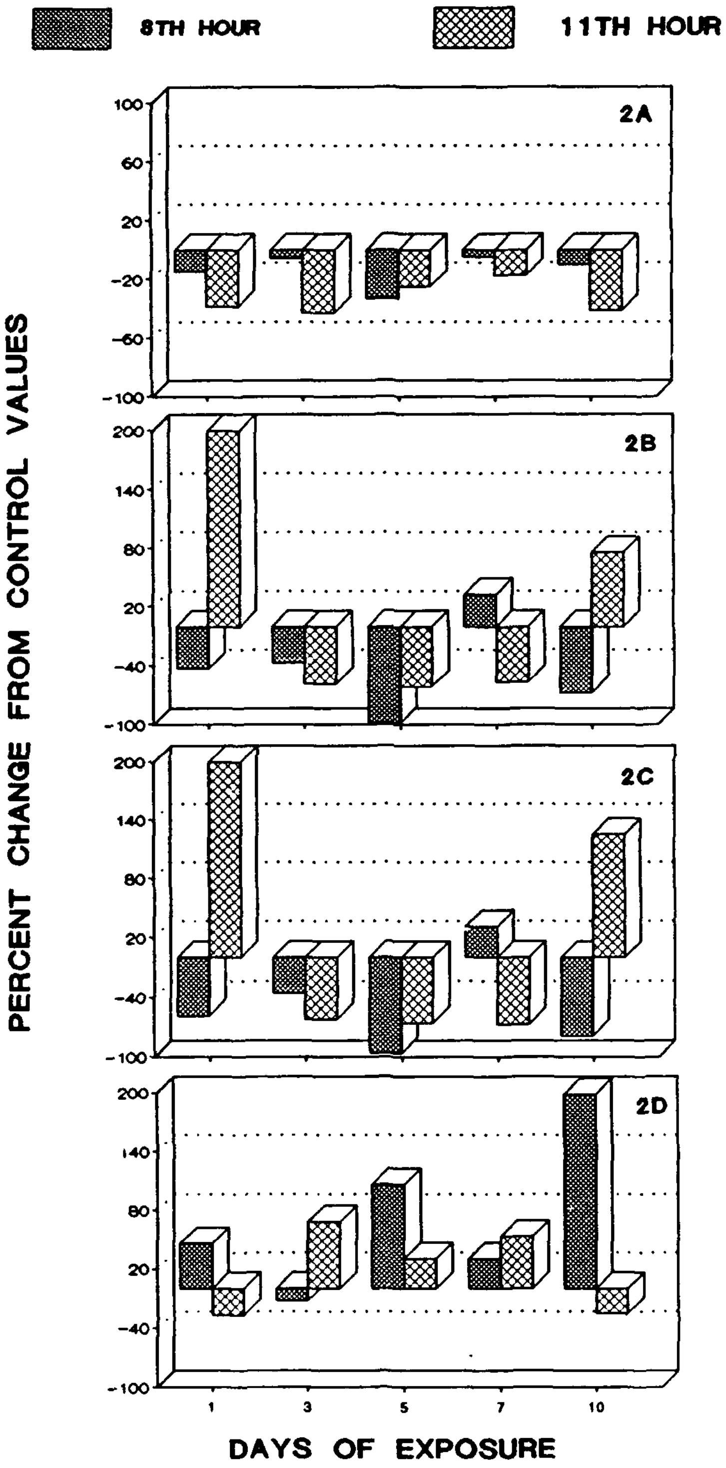
DAYS OF EXPOSURE



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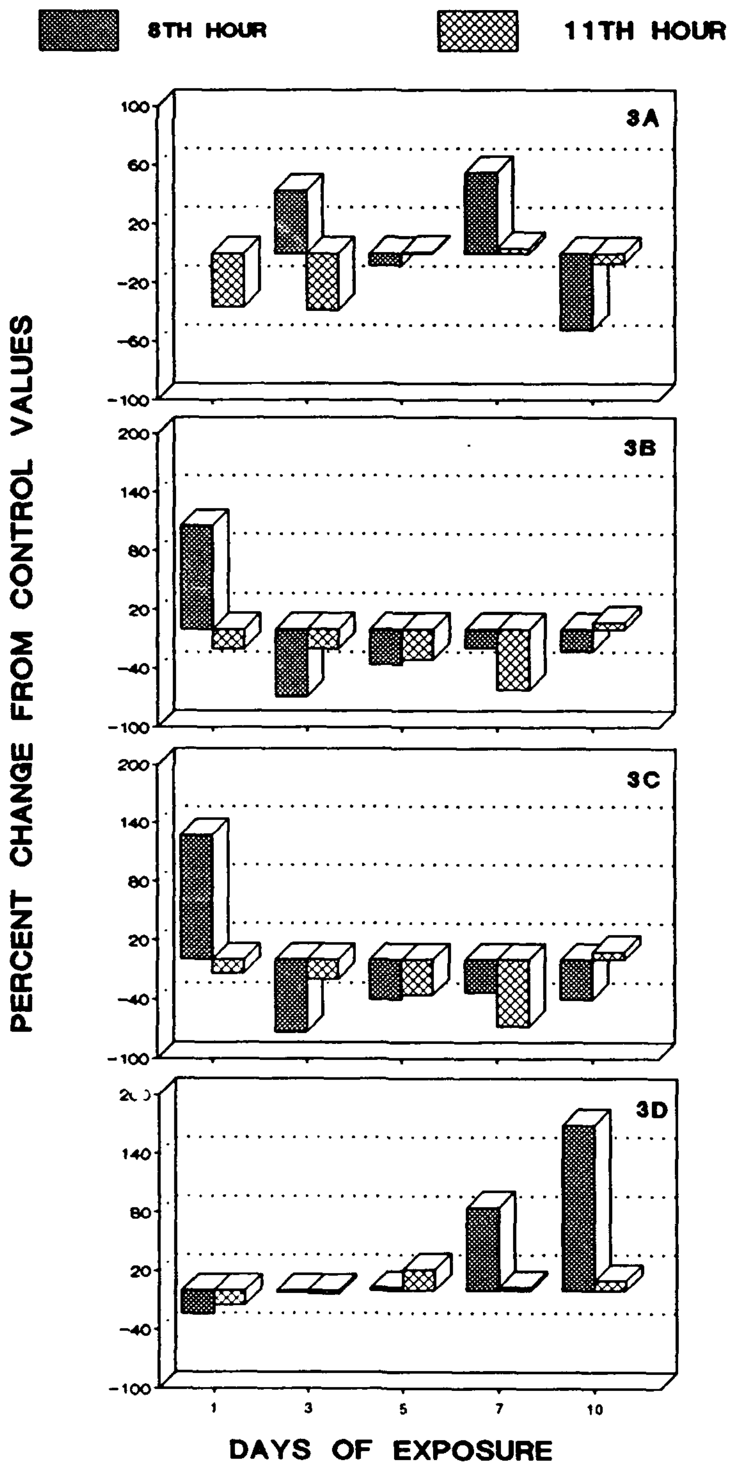

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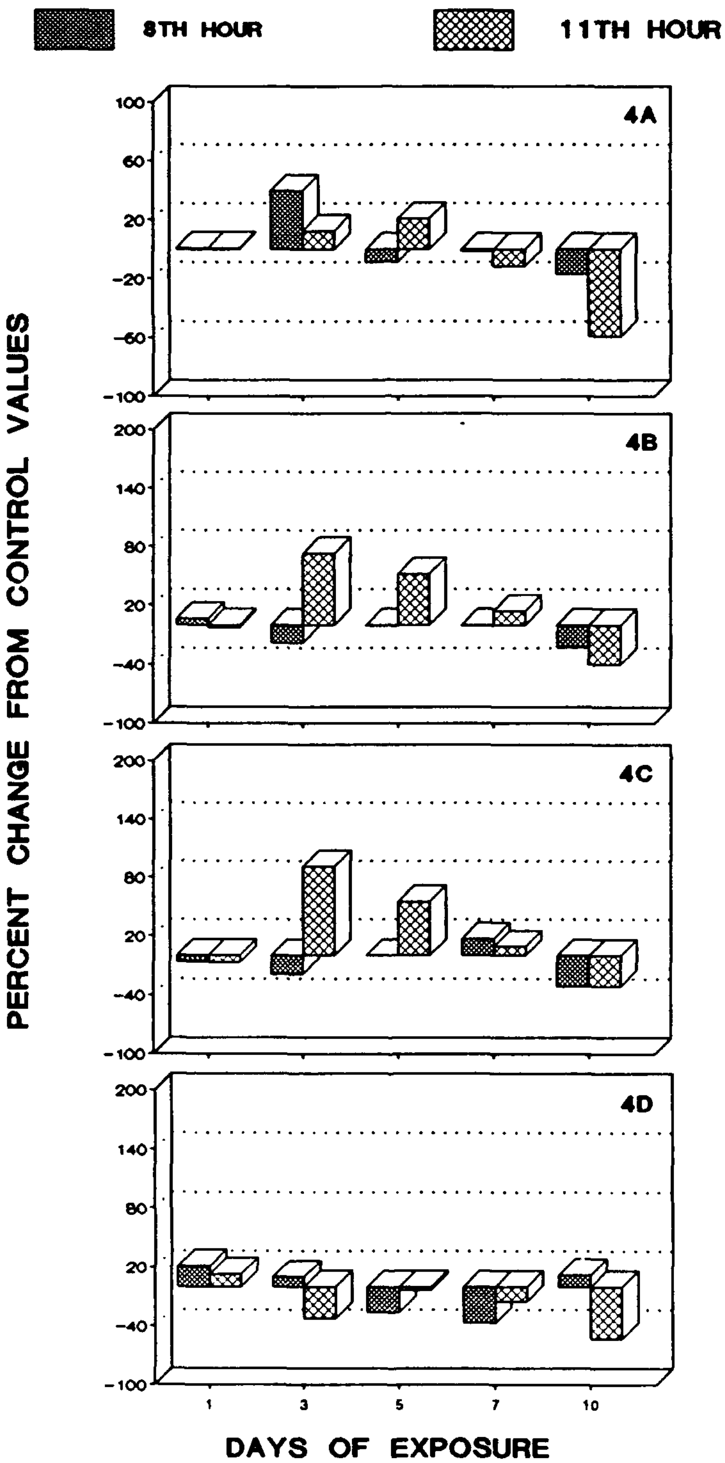
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# PERIODICITY OF LIPID CONTENT AND COMPOSITION IN DIATOMS

## INTRODUCTION

Previous studies in our lab (Sicko-Goad et al. 1988, 1989a,b,c,d) showed that lipid content and fatty acid composition in *Cyclotella meneghiniana* varied during the course of a 16:8 light/dark cycle, and these studies suggested that lipid content could be responsible for altering effects of lipophilic toxicants. We hypothesized that the toxicity effects observed could be due to:

1. Increased partitioning of the lipophilic toxicant due to increased levels of total extractable lipid, or,
2. Alterations in lipid content, i.e. changes in relative composition of storage or polar lipids.

To test this hypothesis, studies were conducted on three diatoms, *Cyclotella meneghiniana*, *Melosira varians*, and *Stephanodiscus binderanus*, to determine variation in lipid content and composition with changes in the light/dark cycle and temperature during a 24 hour period of logarithmic growth. Preliminary results of these studies are presented here.

## MATERIALS AND METHODS

For gravimetric determination of total extractable lipid, between 75 and 200 ml of culture were withdrawn per sample, filtered onto prewashed and preweighed Gelman A/E glass fiber filter, air dried, then dried in a vacuum at 60° for 24 hours. The filters were then reweighed to determine dry weight, and frozen for subsequent lipid extraction. Concurrent with dry weight analyses, smaller volumes of culture (9 ml) were withdrawn and placed in a tube containing paraformaldehyde-glutaraldehyde at final concentrations of 1% in 0.05 M sodium cacodylate buffer. Cell counts were performed on these samples with either a hemocytometer or plankton counting cell to determine cell densities.

For lipid analysis, the frozen filters were placed in a pre-extracted thimble and extracted in a micro-Soxhlet with chloroform for 12 hours (Orcutt and Patterson 1975). The extract was then concentrated in a Kuderna-Danish flask, evaporated under nitrogen, redissolved in chloroform and separated with a separatory funnel. This extract was dried under nitrogen stream in pre-weighed Teflon lined screw cap amber vials and weighed for total gravimetric lipid. Samples were then flushed under nitrogen and frozen for analyses of the lipid classes.

For lipid class analysis, samples were redissolved in methylene chloride to concentrations of 20 to 50 μg lipid in spotting volumes of 10 to 20 uL. The samples were spotted with Hamilton syringes onto cleaned and blank scanned silica coated chromarods (type SIII), held in a frame and developed and scanned in an Iatroscan Mark IV (TLC-FID, FTID) system using a development system described by Parrish (1986).

The development is three staged, consisting of the following:

1. Developed in 50 ml solution of hexane, diethyl ether, and formic acid (99:1:0.05) for 25 minutes, conditioned for 5 minutes, and redeveloped in the same solvent system for 20 minutes. Rods are then partially scanned for hydrocarbons, wax esters, and ketones.

2. The rods are then reconditioned and developed in 50 ml of a solution of hexane, diethyl ether, and formic acid (80:20:0.1) for 40 minutes. The rods are then again partially scanned for triglycerides, alcohols, and sterols.
3. The third development consists of two 14 minute developments in 100% acetone, followed by two developments in 50 ml of solution containing dichloromethane, methanol, and water (5:4:1). During the last scan, the FTID detector is also used for the additional detection of N in Chla and phospholipids. The rods are scanned their entire length for chlorophyll *a*, acetone-mobile polar lipids, and phospholipids.

Quantitative determinations of lipid class composition are based on dose-response calibration equations generated by analysis of a wide range of concentrations of standards for each lipid class.

## RESULTS

*Cyclotella meneghiniana*

In terms of growth preference, we have shown previously that *C. meneghiniana* prefers long light periods and grows best under the 20:4 L/D regime. The amount of total extractable lipid (TEL) in *C. meneghiniana* was variable both with light regime and temperature. Figures 1 and 2 demonstrate that growth at 20°C under both 16:8 and 20:4 L/D regimes resulted in a bimodal pattern of lipid production. However, the patterns are reversed. Under the 16:8 cycle, the largest percentage of lipid in the cells occurs in the late afternoon, whereas under the 20:4 regime, the largest percentage of lipid occurs in the mid-morning hours.

Temperature has a pronounced effect on total lipid. Under both light regimes at 15°C lipid production is rather constant, with less variation and a lower percent dry weight of lipid occurring under a 16:8 light/ dark regime.

*Melosira varians*

Of the three experimental organisms, *M. varians* exhibits the fewest preferences in terms of growth conditions and was able to grow under a variety of conditions, although growth was maximal under the 12:12 L/D regime. Under all growth regimes tested (Figs. 3-5), there appeared to be a bimodal distribution of lipid at 20°C, although the pattern is least pronounced under the 12:12 L/D cycle.

Like *C. meneghiniana*, lower temperature results in a constant percentage of lipid throughout the day, with approximately the same percentage occurring in both the 16:8 and 20:4 regimes. Data analysis is as yet incomplete for 12:12 L/D at 15°C.

*Stephanodiscus binderanus*

In terms of growth preference, *S. binderanus* is unable to sustain growth at the 20:4 L/D regime, and exhibits maximal growth under the 12:12 regime. At 20°C there is at least a bimodal pattern of lipid production under the 16:8 regime. The pattern of lipid production and accumulation is more complicated during rapid growth under the 12:12 L/D cycle. Total extractable lipid is more constant and is lower at 15°C (Figs. 6-7).

Detailed lipid analysis was performed using the Iatroscan Mark IV system. The lipid extract was analyzed for the following fractions: hydrocarbons, wax esters, ketones, triglycerides, free fatty acids, alcohols, sterols, chlorophyll *a*, acetone mobile polar lipids, and phospholipids. In all diatoms examined so far, the largest fractions by percentage of total lipid are chlorophyll *a* and triglyceride. For ease of comparison, ratios of polar and non-polar lipids in relationship to the major lipid components are presented.

A comparison of the chlorophyll *a* to triglyceride ratio for all three organisms (Fig. 8) demonstrates that at 1400 hrs, all organisms are lowest in chlorophyll *a* and are highest in triglycerides. *Stephanodiscus binderanus* has a maximum chlorophyll peak at approximately 0500 hrs, although all three diatoms are high in chlorophyll during the early morning hours. There appears to be a secondary chlorophyll peak in the evening.

When chlorophyll *a* is compared with all non-polar lipids (Fig. 9) the same trend occurs. Since the low ratios at 1400 hrs are approximately the same in Figs. 8 and 9, it indicates that in all three organisms, the major non-polar lipid is triglyceride.

A comparison of the ratio of non-polar to polar lipids (Fig. 10) demonstrates that *Melosira* is rather constant throughout the day. *Cyclotella* has a maximum peak in non-polar lipids early in the morning. *Stephanodiscus* is highest in non-polar lipids at 8 am, but is relatively constant throughout the remainder of the day.

The trends suggested on the previous board were examined in more detail to determine the contribution of individual lipid fraction to the ratios. For *Cyclotella meneghiniana*, it can be seen (Fig. 11) that the pattern for triglycerides (as a percent of total dry weight) follows the change in the ratio of non-polar to polar lipids, indicating that changes in non-polar lipids are due to changes in the triglyceride component of total lipids. Similarly, we examined the contribution of both chlorophyll *a* and acetone-mobile polar lipids and phospholipids to the ratio of polar to non-polar lipids. Figures 12 and 13 demonstrate that although chlorophyll *a* follows the same pattern as the ratio of all polar to non-polar lipids, both AMPL and phospholipid follow this pattern. Consequently, the ratio is not determined entirely by the chlorophyll contribution.

Examination of similar data sets for *Melosira varians* reveals that although there is some suggestion of agreement with the storage to polar ratio and triglyceride percentage, there is some deviation, especially in the late evening and early morning hours (Fig. 14). Although triglycerides are a major component of the lipid fraction, *Melosira* is also characterized by a high proportion of free fatty acids. The values over a 24 hour period ranged from 1 to 33%. Free fatty acids were not a major component in either *Cyclotella* or *Stephanodiscus*. The contribution of chlorophyll *a* to the polar lipid fraction (Fig. 15) mimics the trend of the ratio. AMPL and phospholipids do not follow the ratio closely (Fig. 16).

Triglyceride concentrations contribute greatly to and explain the trend of the ratio of storage to polar lipids in *S. binderanus* (Fig. 17). Changes in chlorophyll *a* concentrations in *Stephanodiscus* account for the trends in total polar lipids (Fig. 18); AMPL and phospholipids are minor constituents in *Stephanodiscus* (Fig. 19).

## SUMMARY

1. The most predominant lipid fractions occurring in the three diatoms examined were chlorophyll *a* and triglycerides. Triglycerides can often account for 60-80% of total lipids.
2. More changes occur in lipid content over the course of a 24 hr. period of logarithmic growth than during growth in batch culture.
3. In all three diatoms, there was a bimodal pattern of lipid content when cells were grown at 20°C. This pattern of lipid content was not evident under similar light regimes at 15°C, when lipid content was more constant.
4. *Melosira varians* exhibits the most stable chlorophyll *a* to storage ratio throughout the day. Both *Cyclotella* and *Stephanodiscus* have a high chlorophyll content in the early morning hours. All three diatoms have low chlorophyll to storage ratios at 1400 hrs and demonstrate either chlorophyll *a* synthesis or triglyceride utilization at 2100 hrs.

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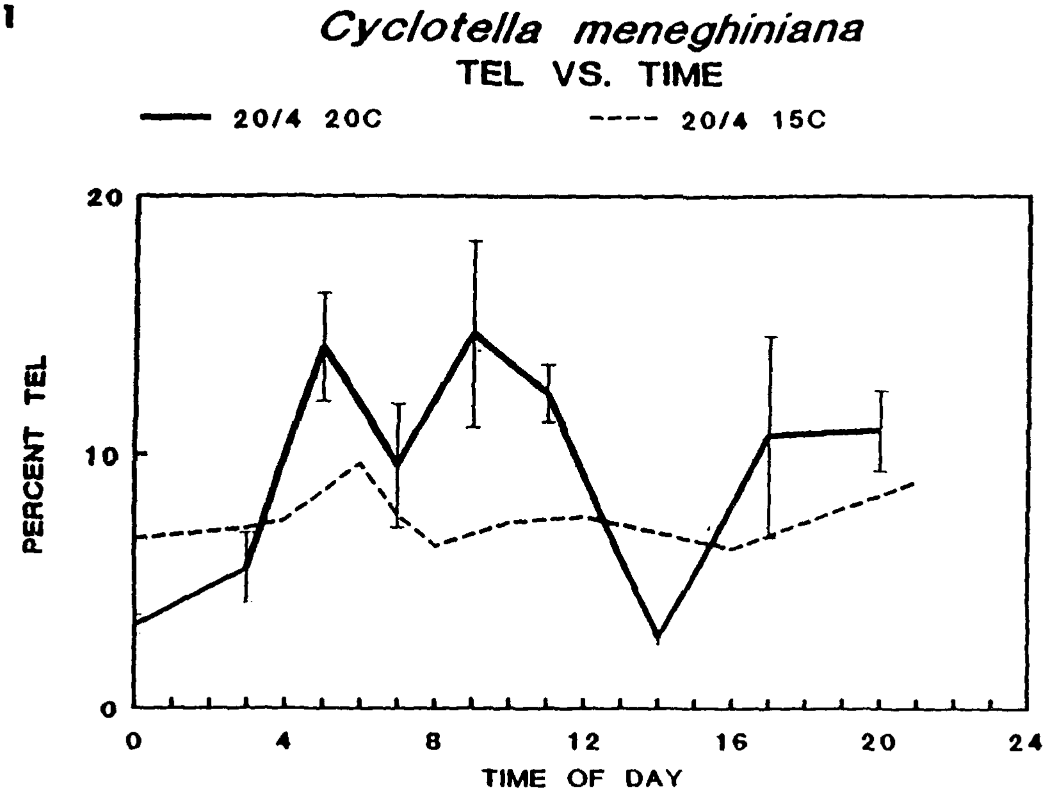
## ACKNOWLEDGMENTS

This work has been supported by grants 88-0315 from the Air Force Office of Scientific Research and R-814194 from the Office of Exploratory Research, U.S.E.P.A.

1

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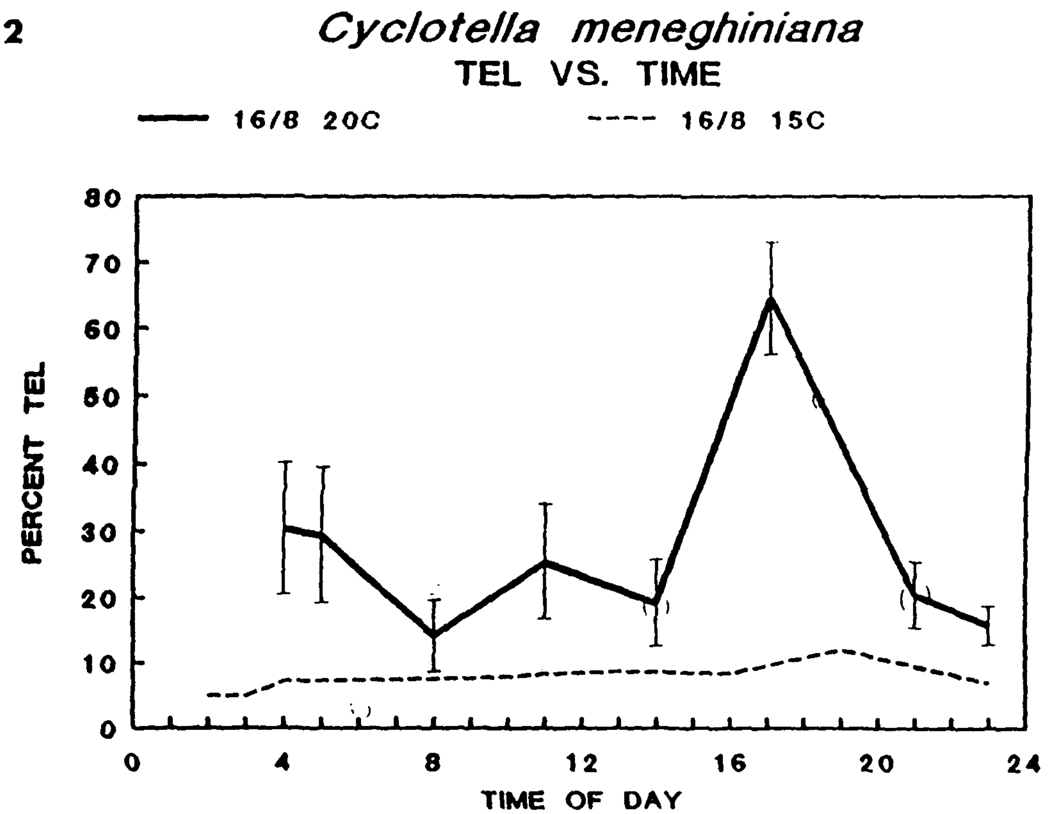
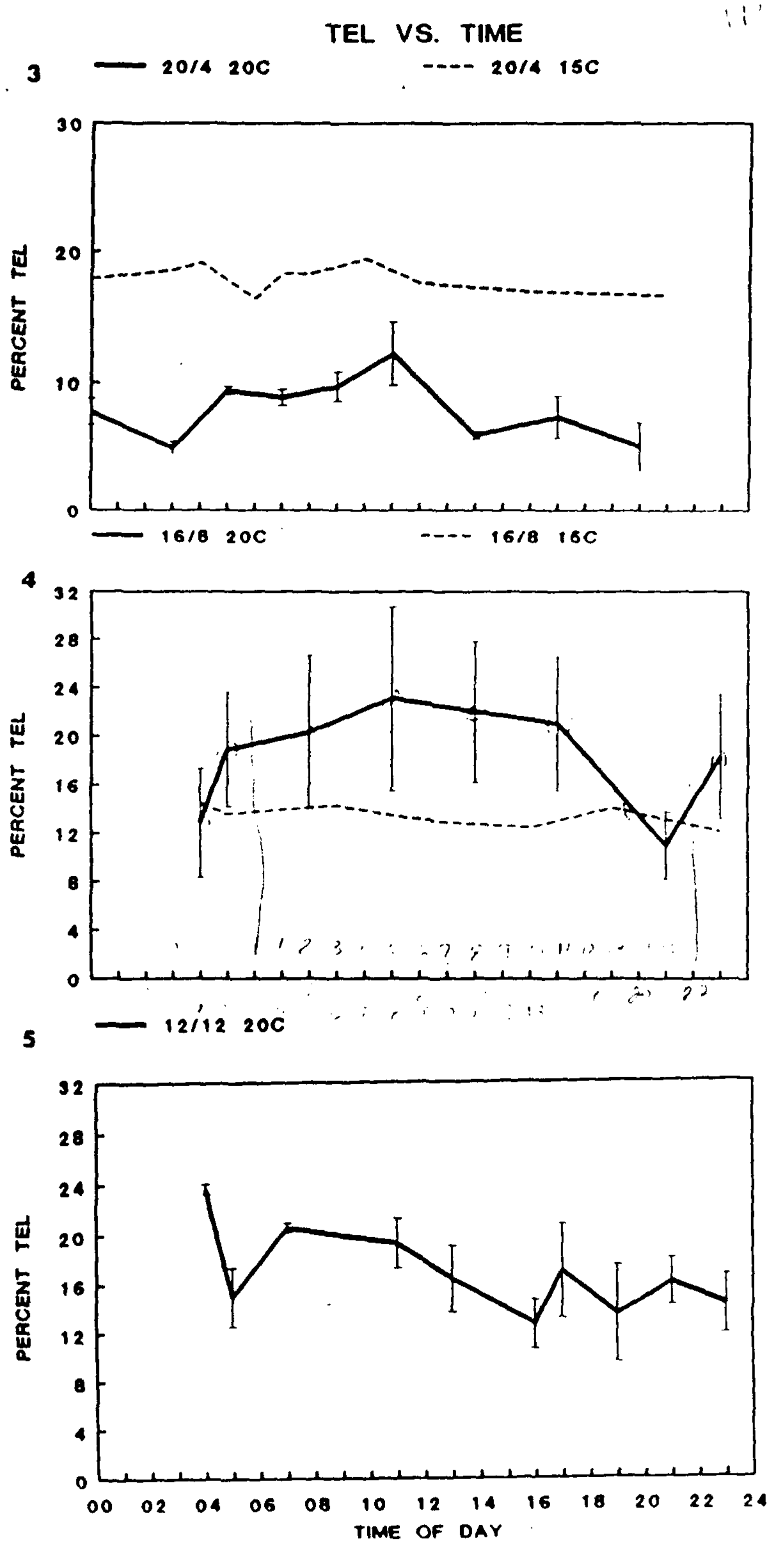



Figures 1-2.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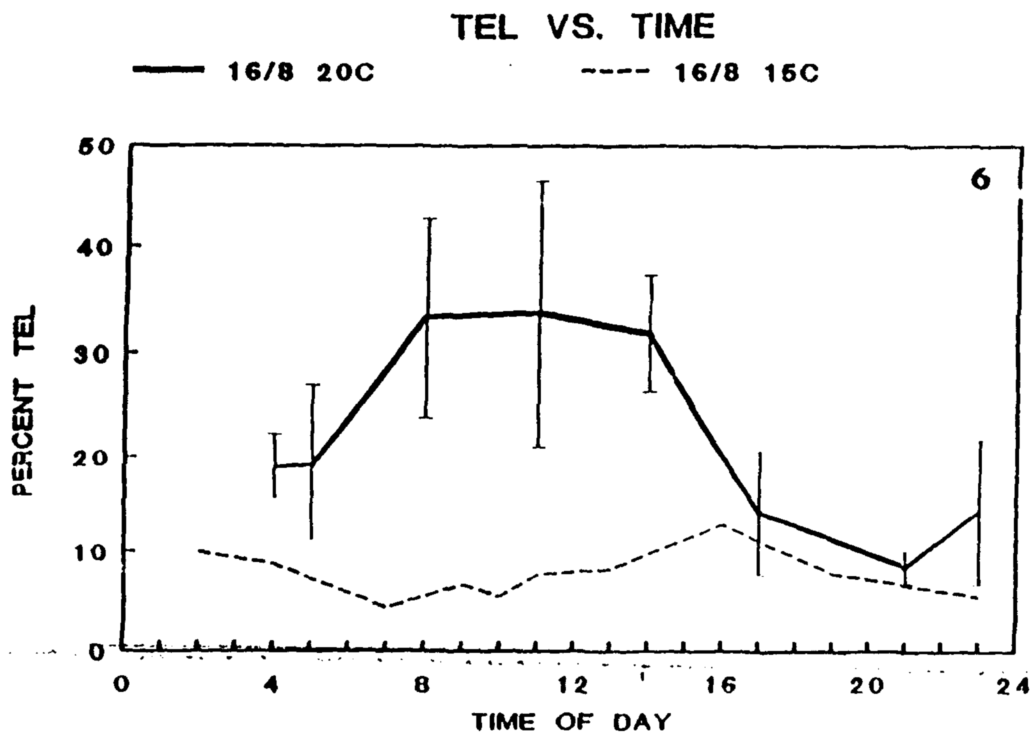
3
20/4 20C
20/4 15C
PERCENT TEL
30
20
10
0
16/8 20C
16/8 15C
4
32
28
24
20
16
12
8
4
0
PERCENT TEL
12/12 20C
5
32
28
24
20
16
12
8
4
0
PERCENT TEL
00 02 04 06 08 10 12 14 16 18 20 22 24
TIME OF DAY

Figures 3-5.


TEL VS. TIME
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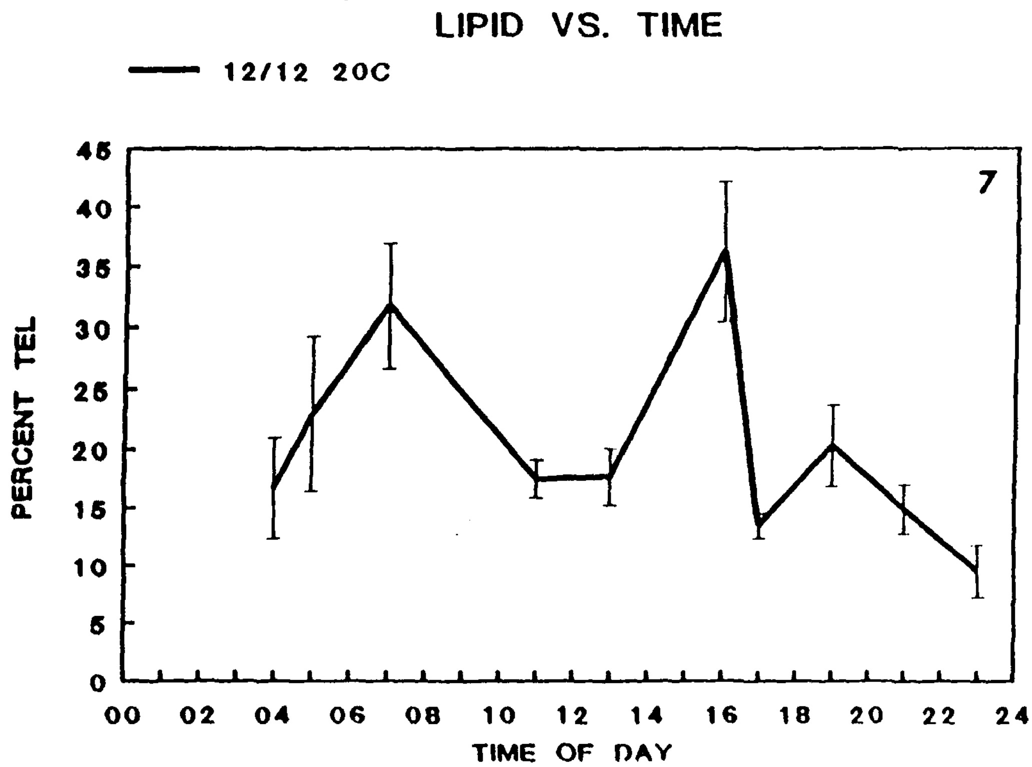
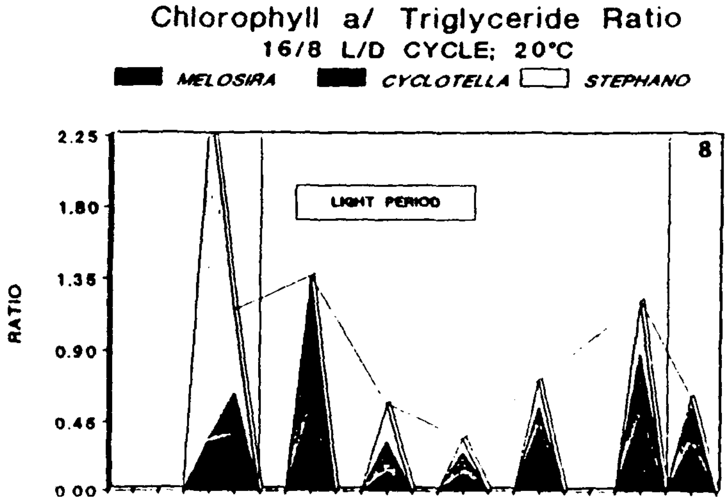
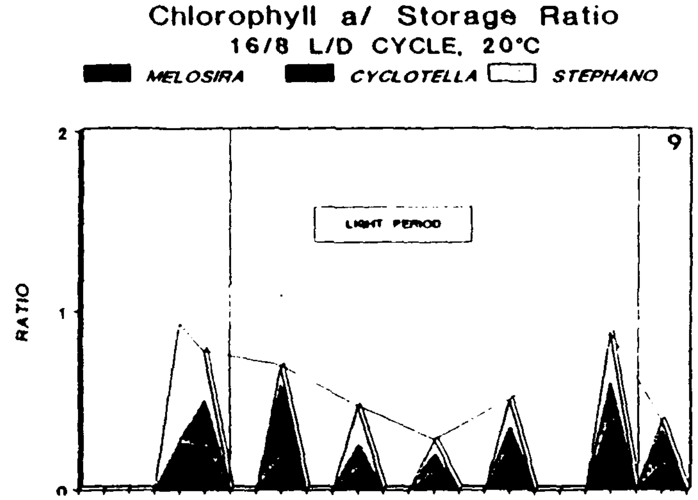
16/8 15C
6
PERCENT TEL
50
40
30
20
10
0
0
4
8
12
16
20
24
TIME OF DAY


LIPID VS. TIME
12/12 20C
7
PERCENT TEL
45
40
35
30
25
20
15
10
5
0
00
02
04
06
08
10
12
14
16
18
20
22
24
TIME OF DAY

Figures 6-7


Chlorophyll a/ Triglyceride Ratio
16/8 L/D CYCLE; 20°C
MELOSIRA
CYCLOTELLA
STEPHANO
LIGHT PERIOD
8
RATIO
2.25
1.80
1.35
0.90
0.45
0.00


Chlorophyll a/ Storage Ratio
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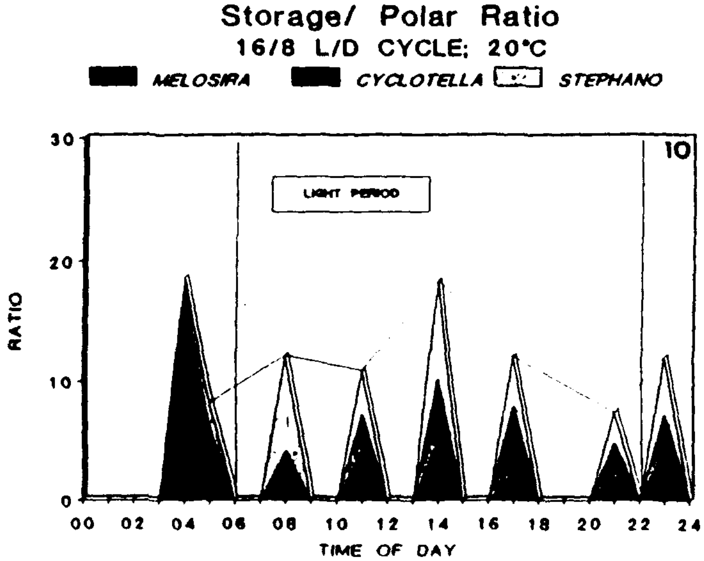
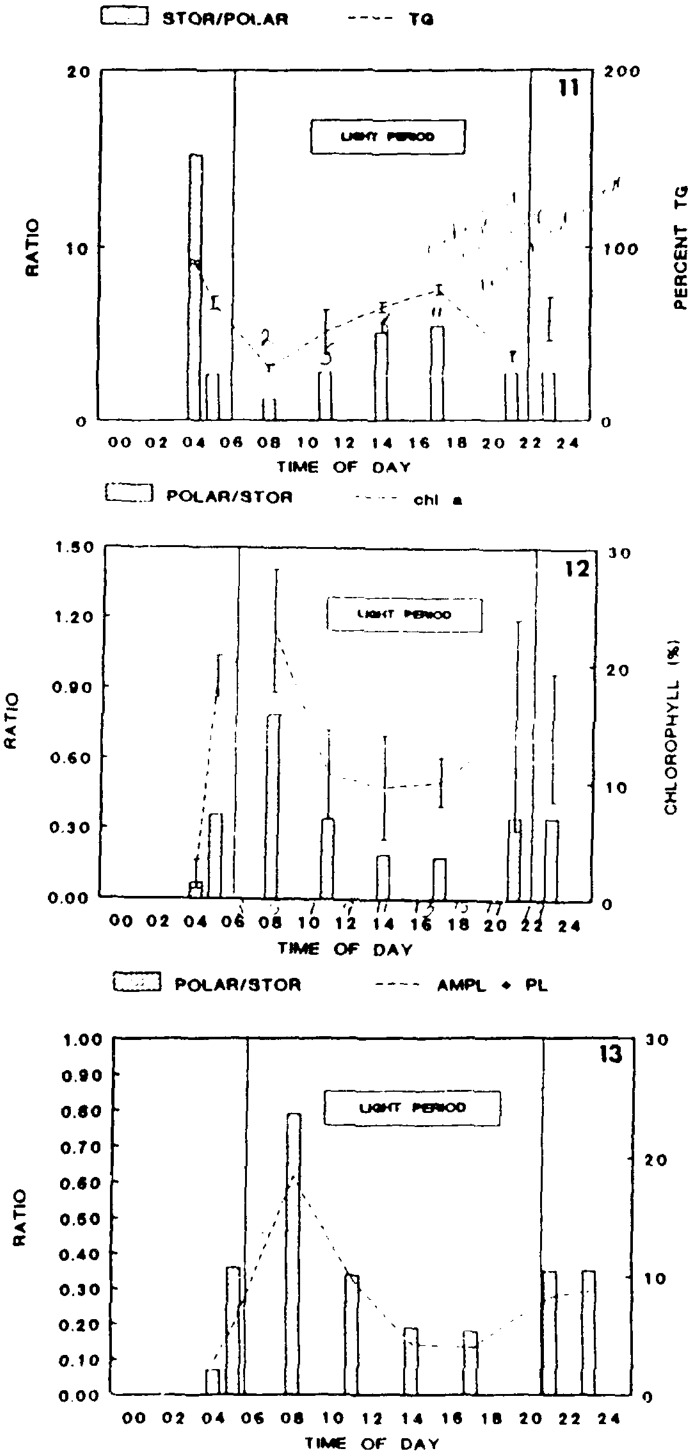
MELOSIRA
CYCLOTELLA
STEPHANO
LIGHT PERIOD
9
RATIO
2
1
0


Storage/ Polar Ratio
16/8 L/D CYCLE; 20°C
MELOSIRA
CYCLOTELLA
STEPHANO
LIGHT PERIOD
10
RATIO
30
20
10
0
00 02 04 06 08 10 12 14 16 18 20 22 24
TIME OF DAY

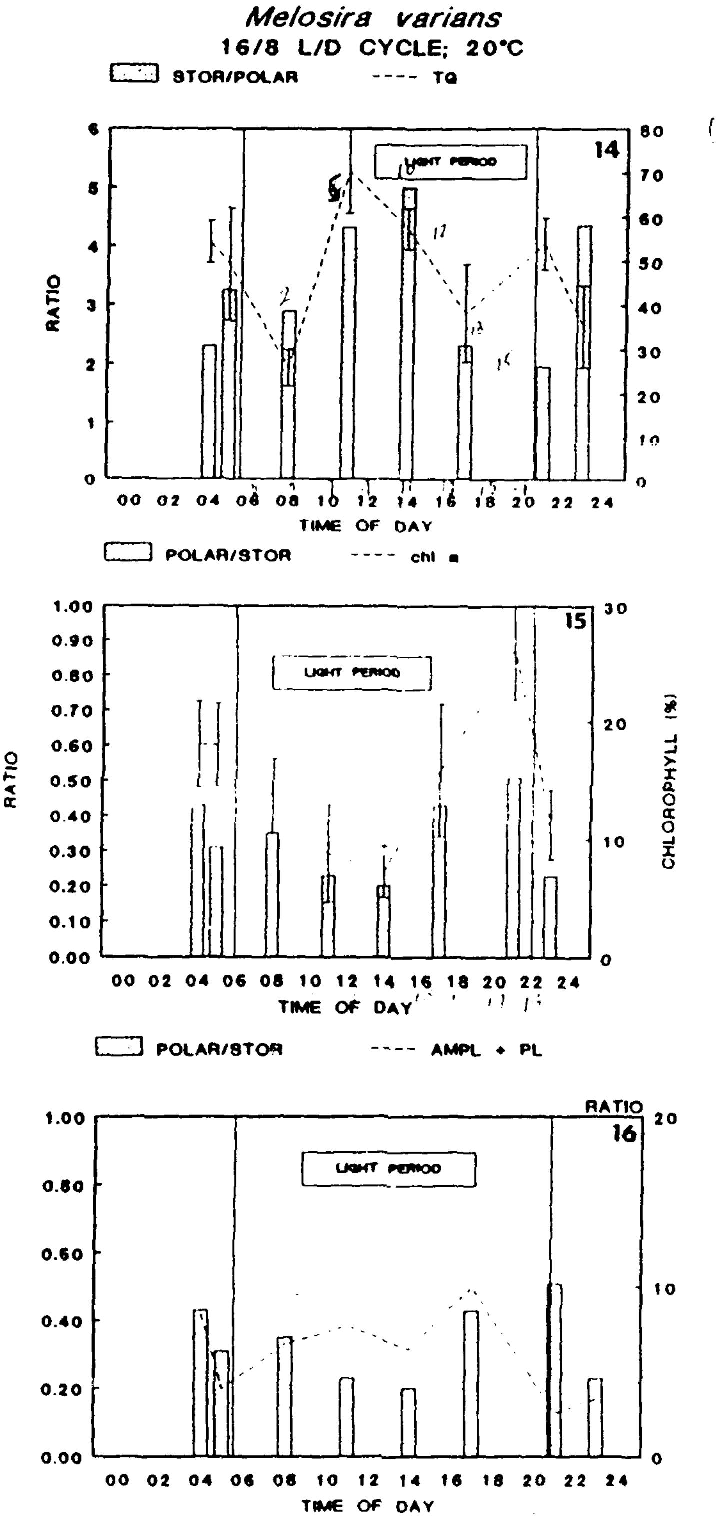
Figures 8-10

# *Cyclotella meneghiniana*

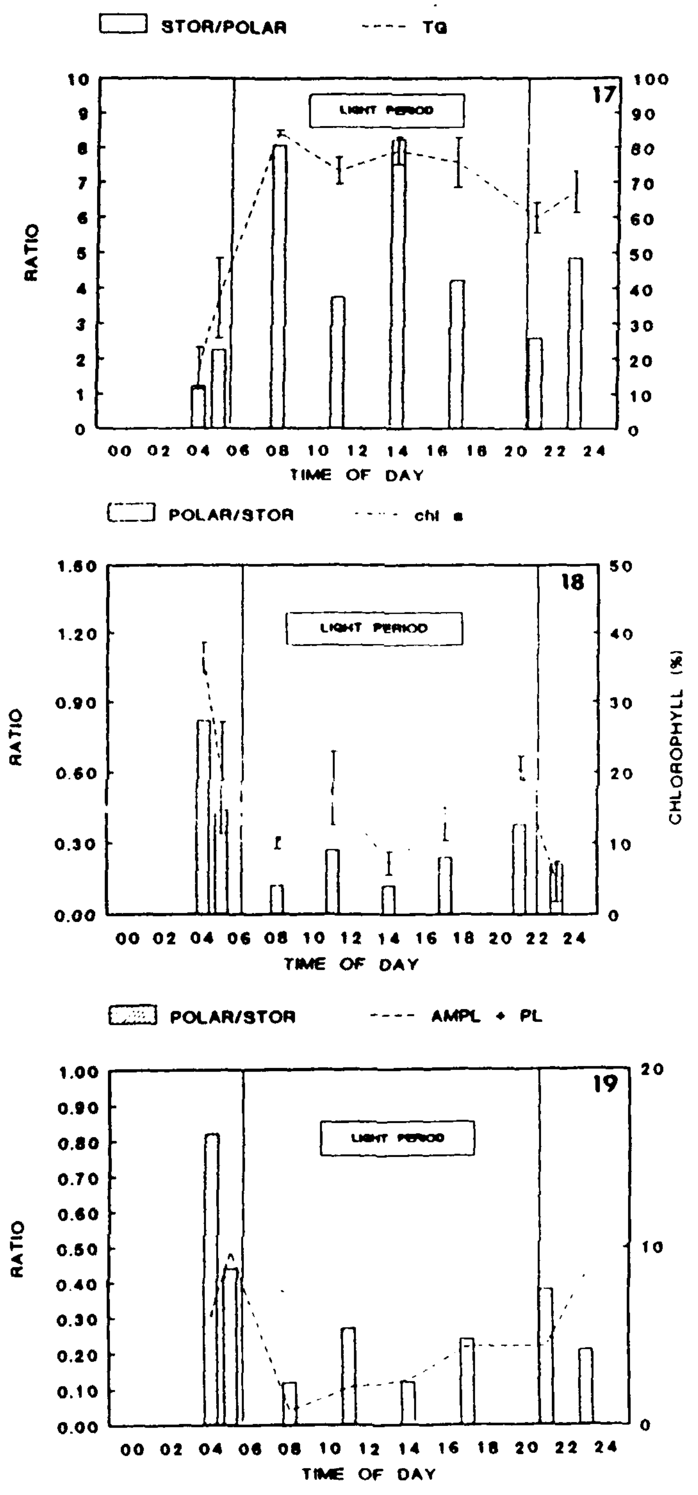
**16/8 L/D CYCLE; 20°C**




Figures 11-13.




Figures 14-16.




Figures 17-19.

## LIST OF FIGURES

# PHOTOPERIOD AND TEMPERATURE EFFECTS ON DIATOM GROWTH

## INTRODUCTION

Previous studies in our laboratory demonstrated that there was a diel periodicity in total lipid and fatty acid composition in the diatom, *Cyclotella meneghiniana*. These changes throughout the day resulted in variable responses upon exposure to lipophilic toxicants. We hypothesized that lipid content and composition were important factors in determining responses to lipophilic compounds and we initiated studies to characterize lipid patterns with changes in environmental variables such as temperature and daylength.

Some of the work we presented last year suggested that several diatoms demonstrated daylength preferences. We have extended these studies and present data which demonstrate that lipid content is variable both with growth cycle and on a diel cycle during exponential growth. Furthermore, trends in lipid composition suggest species specific responses that aid in understanding the distribution of these diatoms in nature.

## MATERIALS AND METHODS

For growth curve studies, algae between 2 and 3 months old (in lag phase growth) were inoculated into fresh WC medium (Guillard 1975) and placed on a shaker table in a walk-in growth chamber which is set at a temperature and light/dark cycle dependent upon the experiment. Prior to introduction of the algae into the new medium with different growth conditions, the batch cultures were acclimated to the temperature and light regime for a period of two weeks to ensure that results were not those of temperature or light shock. Aliquots were withdrawn in duplicate from thoroughly mixed 6 liter flasks 2-3 times a week for a period of approximately 6 weeks. Depending on cell density, between 75 and 200 mL were withdrawn per sample, filtered onto prewashed and preweighed Gelman A/E glass fiber filters, air dried, then dried in a vacuum at 60° for 24 hours. The filters were then reweighed to determine dry weight, and frozen for subsequent lipid extraction. Concurrent with dry weight analyses, smaller volumes of culture (9 mL) were withdrawn and placed in a tube containing paraformaldehyde-glutaraldehyde at final concentrations of 1% in 0.05 M sodium cacodylate buffer. Cell counts were performed on these samples with either a hemocytometer or plankton counting cell to determine cell densities.

For lipid analysis, the frozen filters were placed in a pre-extracted thimble and extracted in a micro-Soxhlet with chloroform for 12 hours (Orcutt and Patterson 1975). The extract was then concentrated in a Kuderna-Danish flask, evaporated under nitrogen, redissolved in chloroform and separated with a separatory funnel. This extract was dried under nitrogen stream in pre-weighed Teflon lined screw cap amber vials and weighed for total gravimetric lipid. Samples were then flushed under nitrogen and frozen for analyses of the lipid classes.

For lipid class analysis, samples were redissolved in methylene chloride to concentrations of 20 to 50 μg lipid in spotting volumes of 10 to 20 μL. The samples were spotted with Hamilton syringes onto cleaned and blank scanned silica coated chromarods (type SIII), held in a frame and developed and scanned in an Iatroscan Mark IV (TLC-FID, FTID) system using a development system described by Parrish (1986).

The development is three staged, consisting of the following:

1. Developed in 50 mL solution of hexane, diethyl ether, and formic acid (98:2:0.05) for 25 minutes, conditioned for 5 minutes, and redeveloped in the same solvent system for 20 minutes. Rods are then partially scanned for hydrocarbons, wax esters, and ketones.
2. The rods are then reconditioned and developed in 50 mL of a solution of hexane, diethyl ether, and formic acid (80:20:0.1) for 50 minutes. The rods are then again partially scanned for triglycerides, FFA alcohols, and sterols.
3. The third development consists of two 15 minute developments in 100% acetone, followed by two 10 minute developments in 50 mL of solution containing dichloromethane, methanol, and water (5:4:1). During the last scan, the FTID detector is also used for the additional detection of N in Chla and phospholipids. The rods are scanned their entire length for chlorophyll *a*, acetone-mobile polar lipids, and phospholipids.

Quantitative determinations of lipid class composition are based on dose-response calibration equations generated by analysis of a wide range of concentrations of standards for each lipid class.

*Melosira varians*

Characterized as a taxon of eutrophic waters, *Melosira varians* is a littoral form with a broad range of tolerances to various parameters. It is considered a facultative plankter by Cholnoky (1968), however it has not been found in the open waters of the eutrophic Laurentian Great Lake Erie. Rather it may occur in the nearshore plankton of the Great Lakes in waters which are eutrophied (Stoermer and Yang 1969). It has a pH optimum of 8.5 with a range of 6.4–9 (Lowe 1974). It is recorded as being able to olerate osmotic stress, become common in brackish water, and it is suggested as being a facultative nitrogen heterotroph (Cholnoky 1968).

*Melosira varians* demonstrates a preference for a short photoperiod. Under long day conditions (20:4 L/D) the taxon appears to maintain itself with cell numbers relatively constant and lipid content varying only slightly (Fig. 1). As the photoperiod is shortened to 16:8 L/D a slight growth pattern is noted and corresponding changes in lipid ontent appear (Fig. 2). When temperature is changed no pattern emerges suggesting this combination of photoperiod and temperature do not favor growth (Fig. 4). The above conditions permit *M. varians* to exist but not as a major constituent of the assemblage. Short days (12:12 L/D) provide conditions in which the characteristic sigmoidal growth pattern is observed (Fig. 3). Under the defined conditions in our experiments photoperiod is a major factor in the capacity of *M. varians* to increase in abundance.

When the daily cycle of lipid production is investigated interesting patterns emerge. During various photoperiods the daily lipid cycle shows little variation at either 20°C or 15°C. Lipid production is constant with only hints of the bimodal cycle present in other taxa (Figs. 5-7).

Diel averages for lipid class composition on selected ratios of classes in *Melosira varians* are presented in Table 1. Under all growth conditions, lipid class compositions and ratios are relatively constant. Chlorophyll *a* concentration is significantly lower when cells are grown under a 16:8 L/D regime at 15°C. Lipid class omposition is most similar under the 12:12 h L/D (20°C) and 20:4 h L/D (15°C) regimes, with chlorophyll *a* to neutral lipid ratios high, chlorophyll *a* concentration high, and moderately low neutral/polar lipid ratios.

*Cyclotella meneghiniana*

*Cyclotella meneghiniana* is a taxon of enriched waters which exhibits a broad range of tolerance to environmental variables. It is a warm water taxon (Stoermer and Ladewski 1976) which primarily is found in the littoral or benthic community of lakes and rivers. Its pH range is 6.4-9 with its optimum at 8.0-8.5 (Lowe 1974). Cholnoky (1968) states it will tolerate osmotic stress and appears to be a facultative Nitrogen heterotroph. It occurs in brackish water and shows a strong halophilic preference (Stoermer and Ladewski 1976, Lowe 1974).

TABLE 1. Mean and standard error for sample lipid fractions and ratios under specified growth conditions. Diel averages, logarithmic growth.

| | *Melosira varians* | | | | |
|---|---|---|---|---|---|
| | Growth condition | | | | |
| | 12:12 (20°C) | 16:8 (20°C) | 16:8 (15°C) | 20:4 (20°C) | 20:4 (15°C) |
| Aliphatic hydrocarbons | 2.0 (0.1) | 3.7 (0.4) | 0.4 (0.1) | 1.0 (0.2) | 0.2 (0.05) |
| Wax and sterol esters | 4.2 (0.4) | 1.6 (0.2) | 5.1 (0.3) | 6.8 (0.6) | 6.5 (0.6) |
| Triglyceride/Free fatty acids | 60.7 (1.7) | 61.6 (2.1) | 68.7 (0.6) | 66.0 (1.8) | 61.9 (2.4) |
| Free alcohol | 0.5 (0.2) | 9.8 (2.3) | 0.4 (0.1) | 0.7 (0.2) | 0.6 (0.2) |
| Free sterol | 7.6 (0.4) | 4.0 (0.4) | 8.6 (0.3) | 2.2 (0.3) | 6.0 (0.5) |
| Chlorophyll *a* | 15.7 (0.9) | 13.5 (1.6) | 5.0 (0.3) | 10.3 (0.9) | 12.4 (1.0) |
| Acetone-mobile polar lipids | 1.5 (0.1) | 3.9 (0.6) | 3.8 (0.3) | 3.1 (0.4) | 2.0 (0.3) |
| Phospholipids | 7.8 (0.7) | 1.9 (0.2) | 7.0 (0.5) | 10.0 (0.9) | 10.5 (0.9) |
| Total polar lipid | 25.0 (1.3) | 19.3 (1.4) | 16.0 (0.5) | 23.3 (1.4) | 24.9 (1.8) |
| Total neutral lipid | 75.0 (1.3) | 80.7 (1.4) | 84.0 (0.5) | 76.7 (1.4) | 75.1 (1.8) |
| Chlorophyll *a*/neutral | 0.22 (0.02) | 0.18 (0.03) | 0.06 (0.0) | 0.14 (0.01) | 0.18 (0.02) |
| Neutral/polar | 3.5 (0.3) | 5.1 (0.6) | 5.5 (0.2) | 4.0 (0.3) | 3.5 (0.3) |

TABLE 2. Mean and standard error for sample lipid fractions and ratios under specified growth conditions. Diel averages, logarithmic growth.

| | *Cyclotella meneghiniana* Growth condition | | | |
|---|---|---|---|---|
| | 16:8 (20°C) | 16:8 (15°C) | 20:4 (20°C) | 20:4 (15°C) |
| Aliphatic hydrocarbons | 5.6 (1.4) | 2.3 (0.1) | 2.5 (0.3) | 1.6 (0.2) |
| Wax and sterol esters | 2.0 (0.7) | 15.4 (0.9) | 14.7 (1.2) | 13.6 (1.2) |
| Triglyceride/Free fatty acids | 60.8 (3.7) | 45.6 (1.6) | 46.5 (2.2) | 46.8 (1.7) |
| Free alcohol | 7.7 (2.6) | 1.2 (0.2) | 0.5 (0.2) | 0.6 (0.1) |
| Free sterol | 2.6 (0.4) | 7.0 (0.4) | 4.3 (0.5) | 4.0 (0.4) |
| Chlorophyll *a* | 13.6 (1.8) | 15.4 (1.0) | 18.4 (1.5) | 21.3 (1.0) |
| Acetone-mobile polar lipids | 4.0 (0.7) | 2.9 (0.3) | 3.0 (0.4) | 0.6 (0.05) |
| Phospholipids | 3.6 (0.5) | 10.0 (0.5) | 10.0 (0.9) | 11.2 (0.6) |
| Total polar lipid | 21.2 (2.4) | 28.3 (0.9) | 31.4 (1.7) | 33.1 (1.3) |
| Total neutral lipid | 78.8 (2.4) | 71.7 (0.9) | 68.6 (.7) | 66.9 (1.3) |
| Chlorophyll *a*/neutral | 0.20 (0.03) | 0.22 (0.02) | 0.50 (0.06) | 0.51 (0.05) |
| Neutral/polar | 6.4 (1.1) | 2.7 (0.1) | 2.7 (0.3) | 2.2 (0.1) |

Photoperiod exerts a significant influence on *C. meneghiniana*. It grows under both 16:8 h and 20:4 h L/D regimes but is unable to grow under a 12:12 L/D regime (Figs. 8-10). Total extractable lipid (TEL) is more variable on a diel cycle at 20° than at 15°C (Figs. 11-12). Diel averages of lipid class composition and selected ratios are shown in Table 2. It is obvious that either longer days and/or a slightly reduced temperature are more favorable to growth. For example, triglycerides and free fatty acids are relatively constant under the following regimes: 16:8 (15°C), 20:4 (20°C), 20:4 (15°C). However, they are elevated by about 30% under a regime of 16:8 h L/D at 20°C. Similarly, all indicators of less than optimal growth, i.e., low chlorophyll *a*, low total are grown under this latter regime.

*Stephanodiscus binderanus*

*Stephanodiscus binderanus* is known from the plankton of eutrophic waters in Europe (Huber-Pestalozzi 1942), North America and may maintain populations in slightly brackish water (Stoermer and Yang 1970). Its presence is usually considered an indicator of pollution. The North American strain appears to prefer water at 8-9°C, however it is present in water warmer than 22°C (Stoermer and Ladewski 1976).

Our studies indicate a preference for short days and for colder temperatures. *Stephanodiscus binderanus* did not grow at 20°C and 20:4 L/D however as the photoperiod is shortened growth improves (Fig. 13). We obtained a sigmoidal growth pattern at 20°C and 12:12 L/D (Fig. 14).

On a daily basis lipid production at 20°C is better than 15°C without the bimodal pattern which is prominent at 12:12 L/D (Figs. 15, 16). *Stephanodiscus binderanus* shows signs of stress at 20°C and 16:8 L/D. Diel average lipid class composition for *Stephanodiscus* is presented in Table 3. Under conditions which are more favorable to growth (i.e., short days and/or colder temperatures, neutral to polar lipid ratios are reduced. Chlorophyll *a* and the chlorophyll *a*/neutral lipid ratio are highest under the short day regime.

## SUMMARY

The information provided by lipid analysis offers insights into the condition of the cell at various combinations of environmental parameters. The study of Stoermer and Ladewski (1976) presented preferred temperatures from the analysis of numerous field samples. In their presentation, many observations lie removed from the preferred optimum (it is understood that temperature is only one of a suite of parameters which influence a cell) and it can be asked why do taxa appear over a wide range and only become dominant at certain times? Lipid analysis does not answer this question but it does provide information on the condition of the cell at defined points and may suggest whether a cell is capable of competing or just surviving.

The taxa considered here demonstrate physiological characteristics which aid in understanding their occurrence in nature.

1. *Melosira varians* shows a preference for short days, and somewhat reduced temperatures. It appears to be capable of growth under a variety of conditions, hence its widespread presence in a littoral environment.

2. *Cyclotella meneghiniana* shows a preference for long days and warm temperature, conditions which occur in the summer.

3. *Stephanodiscus binderanus* in the Laurentian Great Lakes appears to prefer cool water. Our data suggest photoperiod exerts a strong influence on this taxon and it may be more temperature tolerant than the literature suggests.

Table 3. Mean and standard error for sample lipid fractions and ratios under specified growth conditions. Diel averages, logarithmic growth.

| | *Stephanodiscus binderanus* Growth condition 12:12 (20°C) | 16:8 (20°C) | 16:8 (15°C) |
|---|---|---|---|
| Aliphatic hydrocarbons | 3.4 (0.3) | 2.0 (0.4) | 2.8 (0.3) |
| Wax and sterol esters | 7.8 (1.0) | 1.0 (0.5) | 27.2 (1.3) |
| Triglyceride/Free fatty acids | 57.2 (1.8) | 68.9 (3.7) | 36.8 (1.8) |
| Free alcohol | 0.5 (0.1) | 9.1 (2.3) | 1.2 (0.1) |
| Free sterol | 4.4 (0.3) | 2.1 (0.4) | 3.5 (0.2) |
| Chlorophyll *a* | 16.9 (1.0) | 13.4 (1.2) | 11.5 (0.7) |
| Acetone-mobile polar lipids | 1.5 (0.2) | 2.2 (0.5) | 5.7 (0.4) |
| Phospholipids | 8.5 (0.6) | 1.4 (0.3) | 11.3 (0.7) |
| Total polar lipid | 31.2 (1.3) | 19.0 (1.8) | 32.1 (1.2) |
| Total neutral lipid | 68.8 (1.3) | 81.0 (1.8) | 67.9 (1.2) |
| Chlorophyll *a*/neutral | 0.26 (0.02) | 0.17 (0.02) | 0.18 (0.01) |
| Neutral/polar | 2.6 (0.2) | 5.42 (0.61) | 2.6 (0.2) |

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## ACKNOWLEDGMENTS

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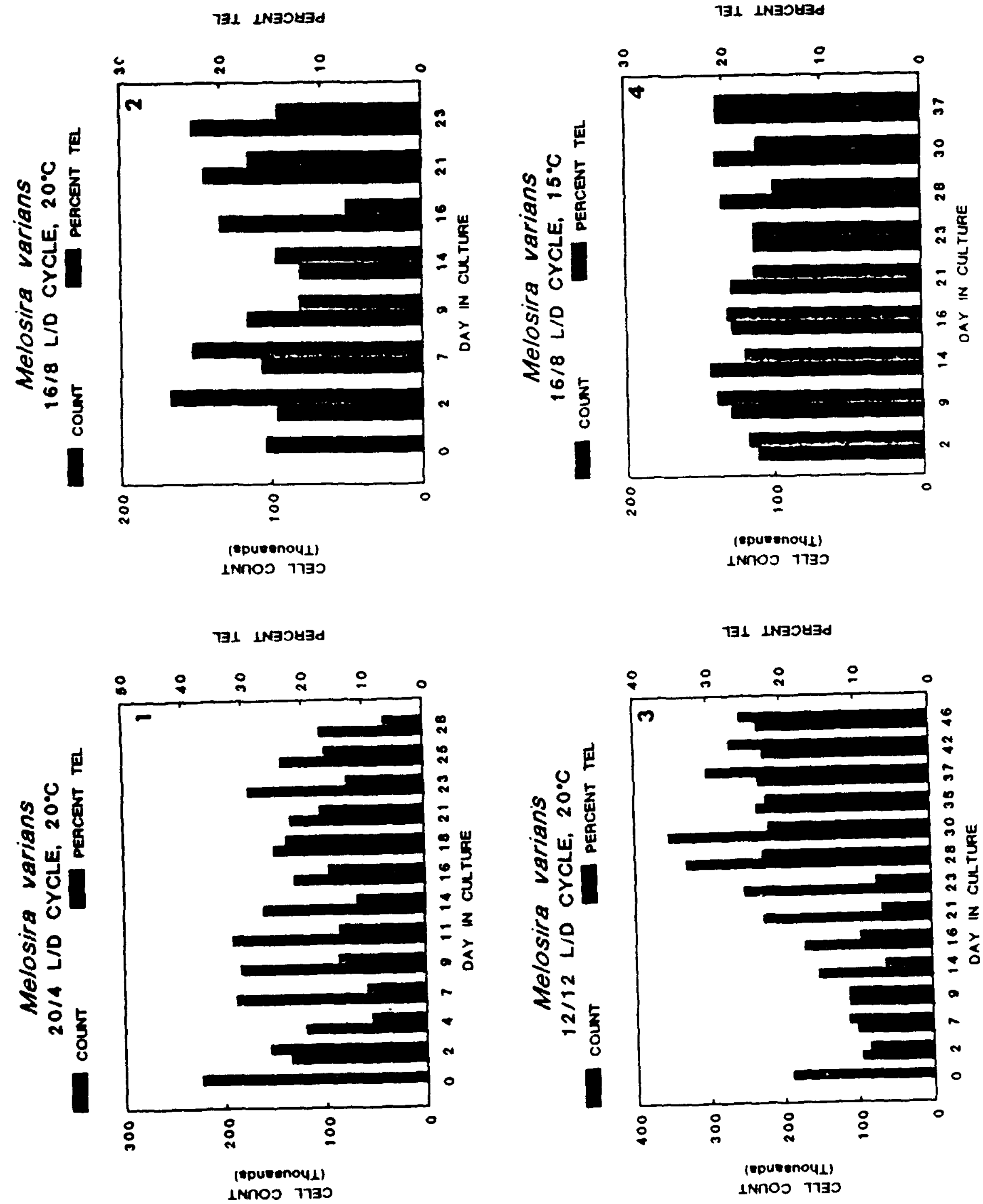
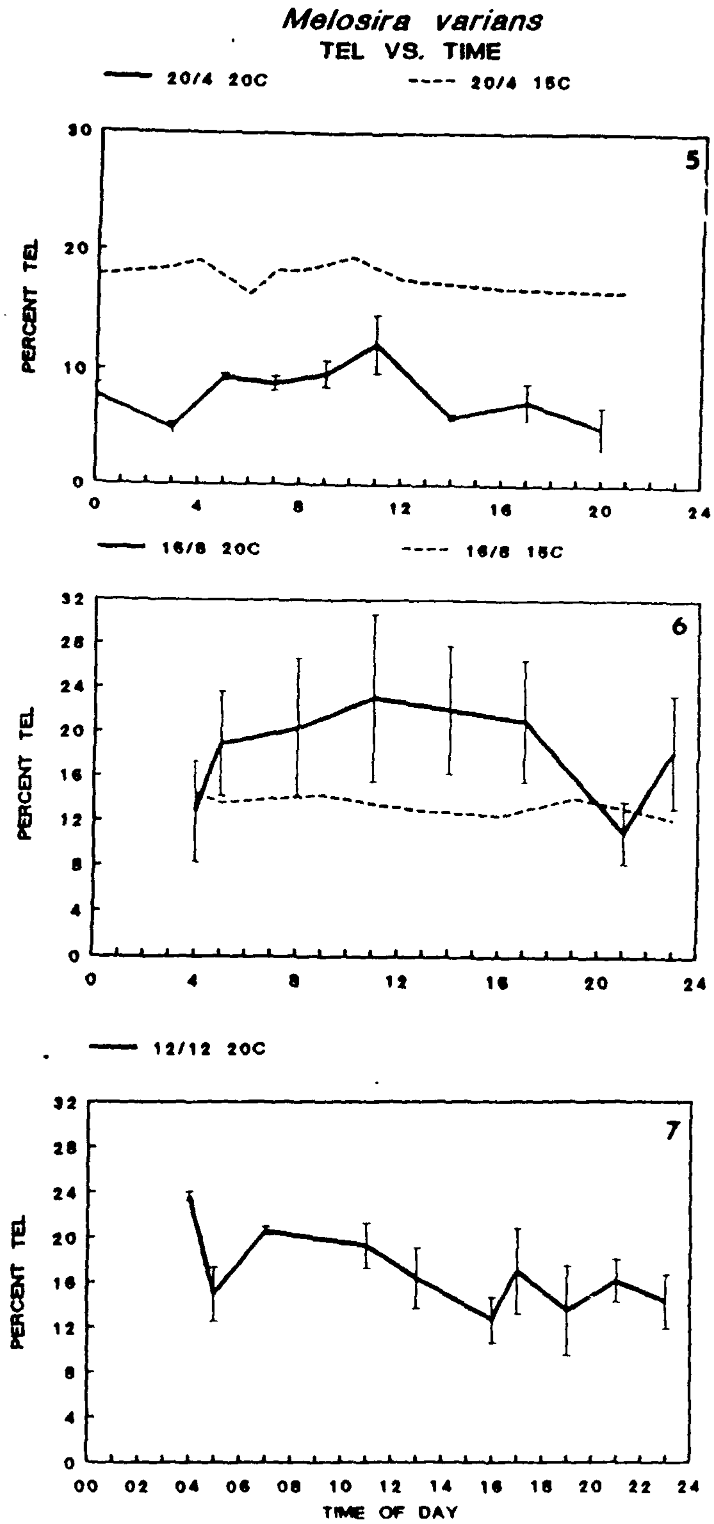
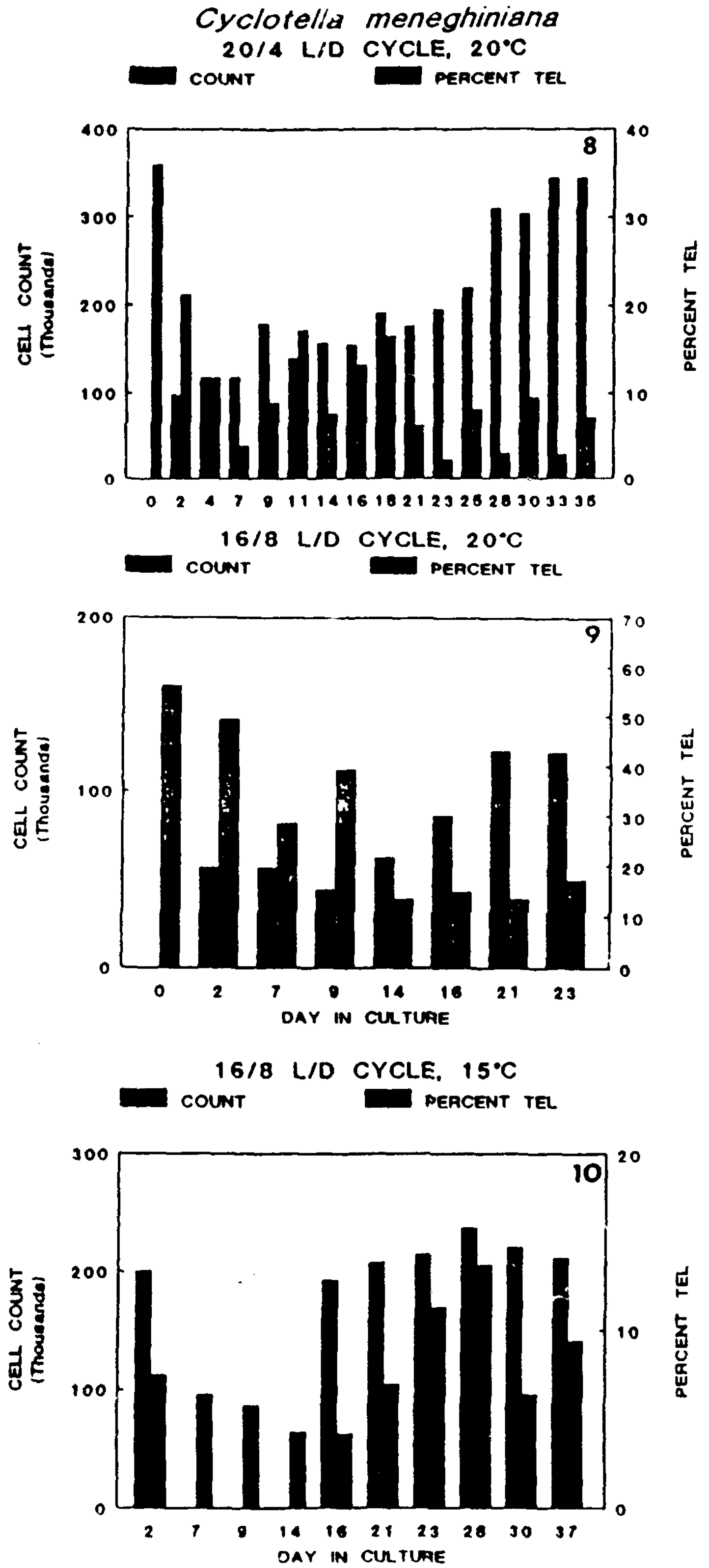


Melosira varians
20/4 L/D CYCLE, 20°C
COUNT
PERCENT TEL
CELL COUNT (Thousands)
DAY IN CULTURE
Melosira varians
16/8 L/D CYCLE, 20°C
Melosira varians
12/12 L/D CYCLE, 20°C
Melosira varians
16/8 L/D CYCLE, 15°C


Figures 1-4.

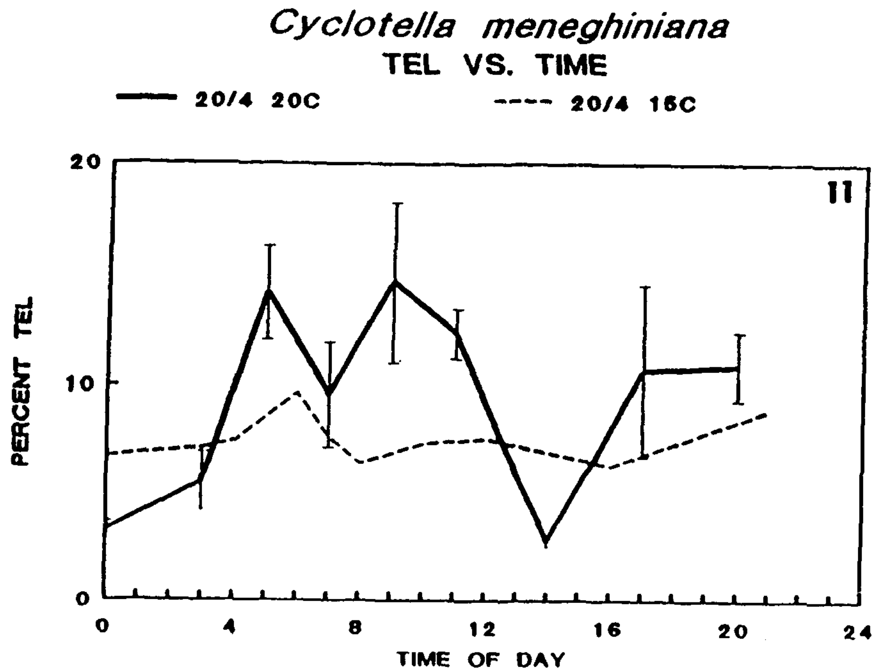

Melosira varians
TEL VS. TIME
20/4 20C
20/4 15C
PERCENT TEL
5
16/8 20C
16/8 15C
6
12/12 20C
7
TIME OF DAY

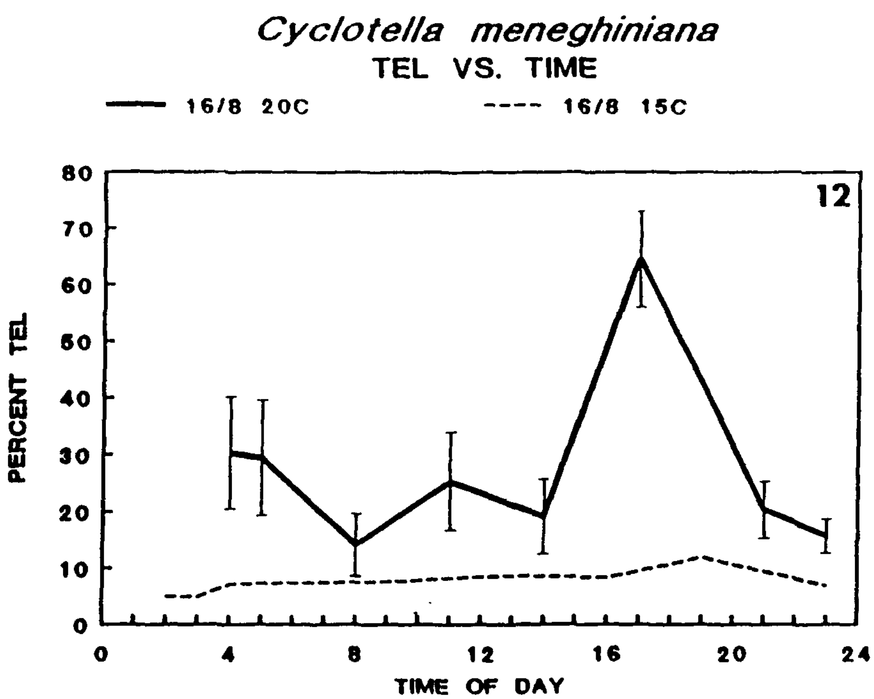
Figures 5-7.



*Cyclotella meneghiniana*
20/4 L/D CYCLE, 20°C
COUNT
PERCENT TEL
CELL COUNT (Thousands)
PERCENT TEL
8
0 2 4 7 9 11 14 16 18 21 23 25 28 30 33 35
16/8 L/D CYCLE, 20°C
COUNT
PERCENT TEL
CELL COUNT (Thousands)
PERCENT TEL
9
0 2 7 9 14 16 21 23
DAY IN CULTURE
16/8 L/D CYCLE, 15°C
COUNT
PERCENT TEL
CELL COUNT (Thousands)
PERCENT TEL
10
2 7 9 14 16 21 23 28 30 37
DAY IN CULTURE


Figures 8-10.




Figures 11-12.

## LIST OF FIGURES

# PHOTOPERIOD AND TEMPERATURE EFFECTS ON DIATOM LIPID COMPOSITION

## I. *CYCLOTELLA MENEGHINIANA*


## ABSTRACT

Previous work in our lab has demonstrated that the diatom *Cyclotella meneghiniana* grows best under long-day conditions. Analysis of the diel patterns and average lipid composition of this diatom grown under light/dark regimes of 16:8h and 20:4h L/D at 15 and 20°C supports the evidence that longer light regimes and/or slightly lower temperatures are optimal for growth. Under a light/dark regime of 20:4h L/D at 15°C, the ratio of neutral to polar lipids is approximately 2.2. Decreasing the duration of light and/or raising the temperature results in an increase in the ratio of neutral to polar lipids to approximately 3. The percentage of chlorophyll and the ratio of chlorophyll *a* to neutral lipids are reduced by shortening the daylength or increasing the temperature. Lipid composition patterns over a diel cycle will be discussed in relationship to the distributional ecology of the organism.


## INTRODUCTION

Previous studies in our laboratory demonstrated that there was a diel periodicity in total lipid and fatty acid composition in the diatom, *Cyclotella meneghiniana*. These changes throughout the day resulted in variable responses upon exposure to lipophilic toxicants. We hypothesized that lipid content and composition were important factors in determining responses to lipophilic compounds and we initiated studies to characterize lipid patterns with changes in environmental variables such as temperature and daylength.

Some of the work we presented last year suggested that several diatoms demonstrated daylength preferences. We have extended these studies and present data which demonstrate that lipid content is variable both with growth cycle and on a diel cycle during exponential growth. Furthermore, trends in lipid composition suggest species specific responses that aid in understanding the distribution of these diatoms in nature.

## MATERIALS AND METHODS

For growth curve studies, algae between 2 and 3 months old (in lag phase growth) were inoculated into fresh WC medium (Guillard 1975) and placed on a shaker table in a walk-in growth chamber which is set at a temperature and light/dark cycle dependent upon the experiment. Prior to introduction of the algae into the new medium with different growth conditions, the batch cultures were acclimated to the temperature and light regime for a period of two weeks to ensure that results were not those of temperature or light shock. Aliquots were withdrawn in duplicate from thoroughly mixed 6 liter flasks 2-3 times a week for a period of approximately 6 weeks. Depending on cell density, between 75 and 200 mL were withdrawn per sample, filtered onto prewashed and preweighed Gelman A/E glass fiber filters, air dried, then dried in a vacuum at 60° for 24 hours. The filters were then reweighed to determine dry weight, and frozen for subsequent lipid

extraction. Concurrent with dry weight analyses, smaller volumes of culture (9 mL) were withdrawn and placed in a tube containing paraformaldehyde-glutaraldehyde at final concentrations of 1% in 0.05 M sodium cacodylate buffer. Cell counts were performed on these samples with either a hemocytometer or plankton counting cell to determine cell densities.

For lipid analysis, the frozen filters were placed in a pre-extracted thimble and extracted in a micro-Soxhlet with chloroform for 12 hours (Orcutt and Patterson 1975). The extract was then concentrated in a Kuderna-Danish flask, evaporated under nitrogen, redissolved in chloroform and separated with a separatory funnel. This extract was dried under nitrogen stream in pre-weighed Teflon lined screw cap amber vials and weighed for total gravimetric lipid. Samples were then flushed under nitrogen and frozen for analyses of the lipid classes.

For lipid class analysis, samples were redissolved in methylene chloride to concentrations of 20 to 50 μg lipid in spotting volumes of 10 to 20 μL. The samples were spotted with Hamilton syringes onto cleaned and blank scanned silica coated chromarods (type SIII), held in a frame and developed and scanned in an Iatroscan Mark IV (TLC-FID, FTID) system using a development system described by Parrish (1986).

The development is three staged, consisting of the following:

1. Developed in 50 mL solution of hexane, diethyl ether, and formic acid (98:2:0.05) for 25 minutes, conditioned for 5 minutes, and redeveloped in the same solvent system for 20 minutes. Rods are then partially scanned for hydrocarbons, wax esters, and ketones.
2. The rods are then reconditioned and developed in 50 mL of a solution of hexane, diethyl ether, and formic acid (80:20:0.1) for 50 minutes. The rods are then again partially scanned for triglycerides, FFA alcohols, and sterols.
3. The third development consists of two 15 minute developments in 100% acetone, followed by two 10 minute developments in 50 mL of solution containing dichloromethane, methanol, and water (5:4:1). During the last scan, the FTID detector is also used for the additional detection of N in Chla and phospholipids. The rods are scanned their entire length for chlorophyll *a*, acetone-mobile polar lipids, and phospholipids.

Quantitative determinations of lipid class composition are based on dose-response calibration equations generated by analysis of a wide range of concentrations of standards for each lipid class.

## RESULTS

*Cyclotella meneghiniana* grew best under long day (20:4h L/D) conditions (Figs. 1-3). Diel averages of lipid class fractions (Table 1) under the growth regimes studied corroborate growth observations. That is, a reduction in temperature or an increase in daylength provide conditions in which neutral/polar lipid ratios are lowest–evidence of increased metabolic activity and synthesis of membrane associated lipids.

Diel patterns of selected lipid classes and ratios are presented for all growth regimes tested (Figs. 4-6). Figure 4 demonstrates that throughout the day, the neutral/polar lipid ratio is significantly lower when the algae are grown under a 20:4h L/D regime. At 20°C, there is an increase in this ratio after the lights come on. The trend is opposite at 15°C. However, there is less diel variation in the ratios when cells are dividing more rapidly (i.e., 20:4h L/D and/or 15°C).

Under conditions of more rapid growth, chlorophyll concentration is much higher (Fig. 5). At 15°C, chlorophyll increases throughout the day and is reduced by approximately 25% in the night. Chlorophyll averages are more uniform throughout the day on a percent composition comparison when cells are grown under a 16:8h L/D 20°C regime.

Under conditions of more rapid growth, triacylglycerol (storage lipids) are reduced and more uniform throughout the day (Fig. 6). Under the 16:8h L/D regime, the lowest percentage of triacylglycerols are found in the latter part of the light period.

## DISCUSSION

Photoperiod exerts a significant influence on *C. meneghiniana*. It grows under both 16:8h and 20:4h L/D regimes, but it is unable to grow under a 12:12 L/D regime. Either longer days and/or a slightly reduced temperature are more favorable to growth. Lipid class percent composition data demonstrate that under conditions favoring more rapid growth, triacyglycerol is reduced in the cells and chlorophyll is increased. Neutral to polar ratios are also reduced, suggesting increased metabolic activity. Under these conditions, lipid classes are more uniform throughout the day and there are no dramatic changes in lipid composition either with the onset of light or dark.

*Cyclotella meneghiniana* is a taxon of enriched waters which exhibits a broad range of tolerance to environmental variables. It is a warm water taxon (Stoermer and Ladewski 1976) which primarily is found in the littoral or benthic community of lakes and rivers. Its pH range is 6.4-9 with its optimum at 8.0-8.5 (Lowe 1974). Cholnoky (1968) states it will tolerate osmotic stress and appears to be a facultative Nitrogen heterotroph. It occurs in brackish water and shows a strong halophilic preference (Stoermer and Ladewski 1976, Lowe 1974).

Although it is reported to be a warm water taxon with a temperature preference of ca. 20°C, the data of Stoermer and Yang (1969) demonstrate maximum number of occurrences in September, which is consistent with slightly cooler water.

A cursory survey of diatom literature reveals *Cyclotella meneghiniana* rare to absent at equatorial latitudes (i.e., more equal LD cycles, Hustedt 1938 and 1949, Foged 1976), increasing in frequency as the distance from the equator increases, present at moderately high latitudes (66N, Foged 1952), present at higher latitudes (70-71N, Foged 1968), and absent at high latitudes (77-80N, Foged 1964). The 66N latitude has 24 hours of light for the last two weeks of June.

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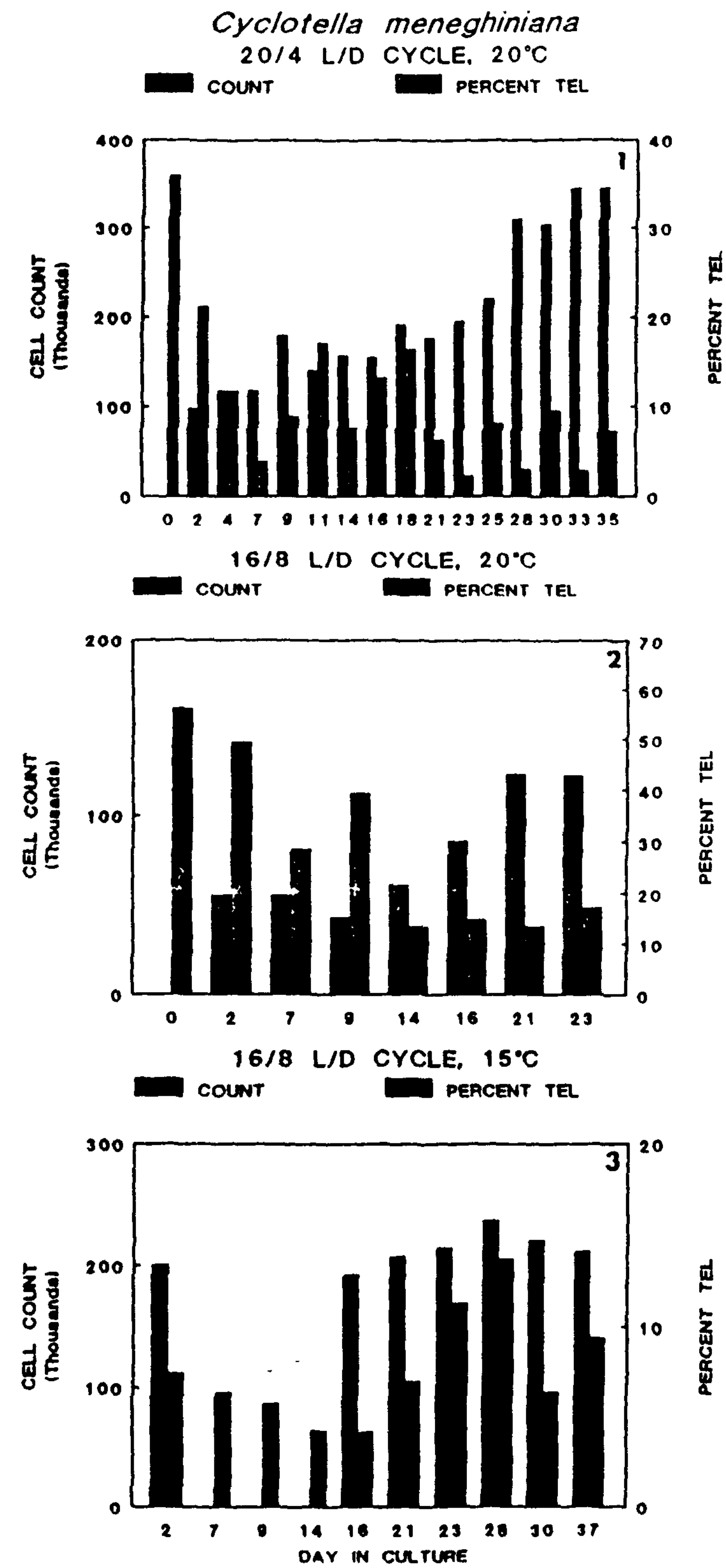
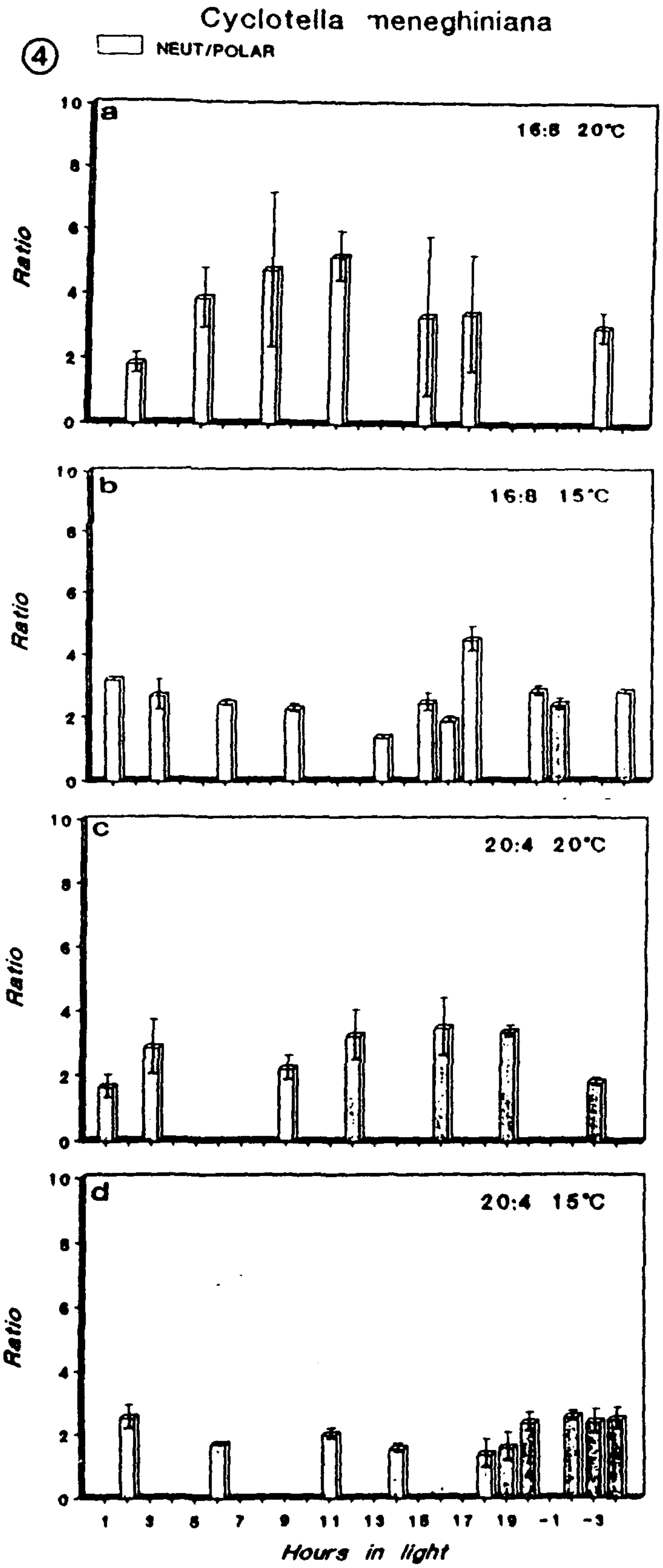
## ACKNOWLEDGMENTS


This work has been supported by grants 88-0315 from the Air Force Office of Scientific Research and R-814194 from the Office of Exploratory Research, U.S.E.P.A.

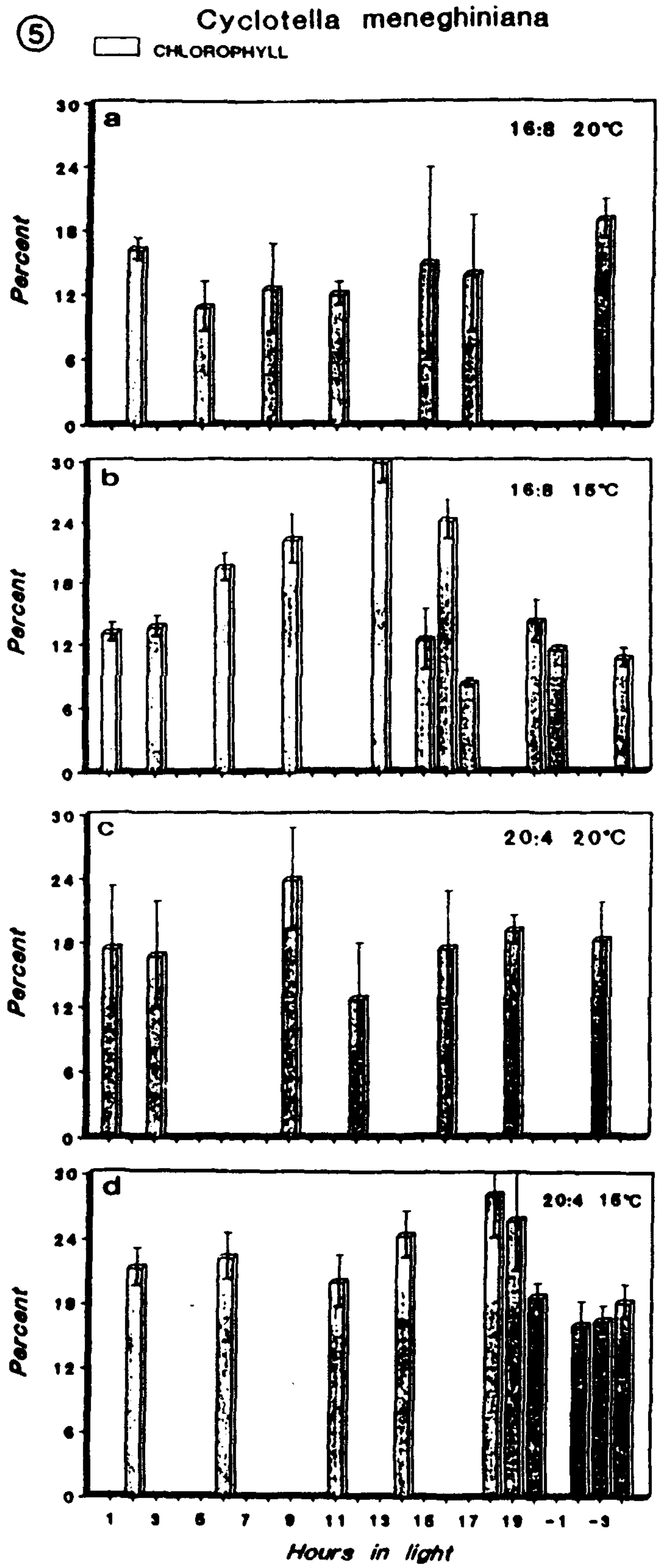

Cyclotella meneghiniana
20/4 L/D CYCLE, 20°C
COUNT
PERCENT TEL
CELL COUNT (Thousands)
PERCENT TEL
0 100 200 300 400
0 10 20 30 40
1
0 2 4 7 9 11 14 16 18 21 23 25 28 30 33 35
16/8 L/D CYCLE, 20°C
COUNT
PERCENT TEL
CELL COUNT (Thousands)
PERCENT TEL
0 100 200
0 10 20 30 40 50 60 70
2
0 2 7 9 14 16 21 23
16/8 L/D CYCLE, 15°C
COUNT
PERCENT TEL
CELL COUNT (Thousands)
PERCENT TEL
0 100 200 300
0 10 20
3
2 7 9 14 16 21 23 28 30 37
DAY IN CULTURE

Figures 1-3.



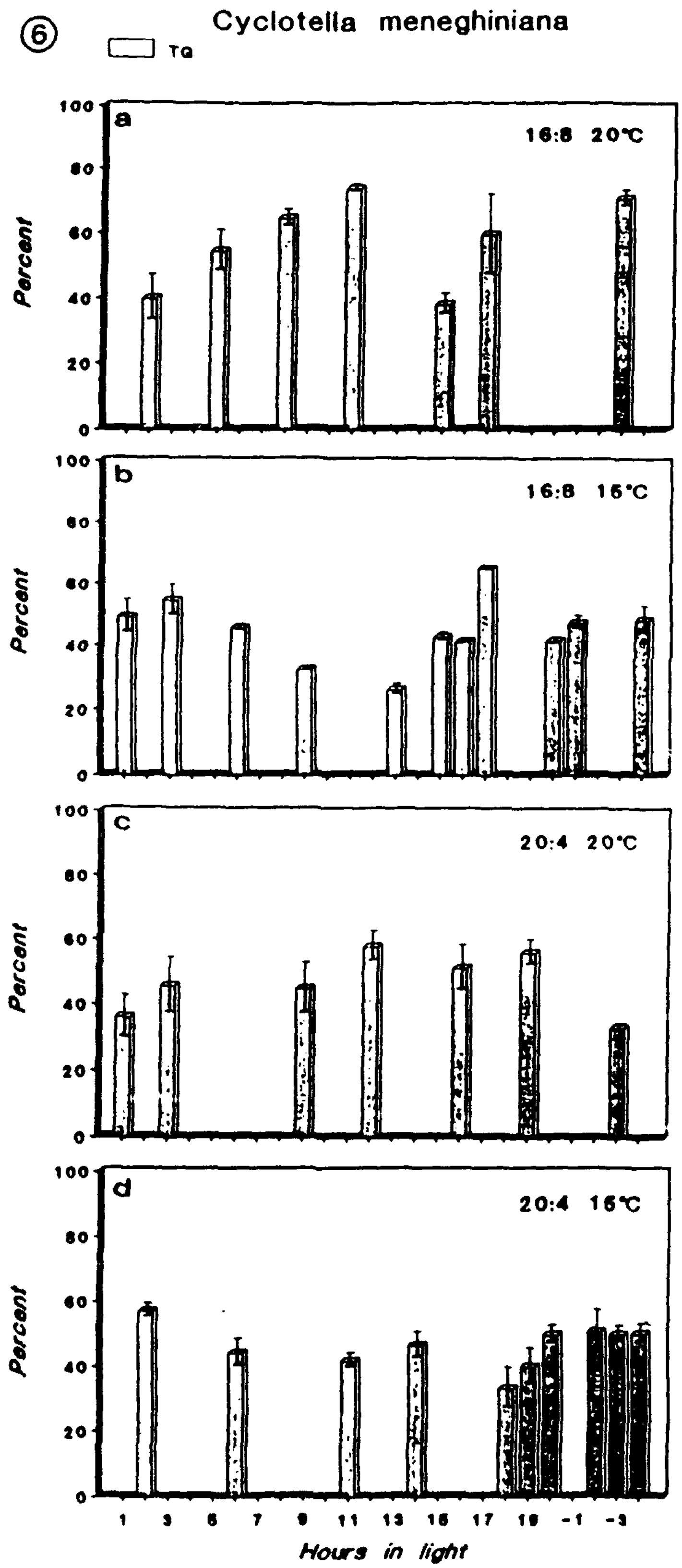
Cyclotella meneghiniana
4
NEUT/POLAR
a
16:8 20°C
b
16:8 15°C
c
20:4 20°C
d
20:4 15°C
Ratio
0
2
4
6
8
10
1
3
5
7
9
11
13
15
17
19
-1
-3
Hours in light


Figure 4a-d.



⑤
Cyclotella meneghiniana
CHLOROPHYLL
a
16:8 20°C
b
16:8 15°C
c
20:4 20°C
d
20:4 15°C
Percent
0
6
12
18
24
30
1
3
5
7
9
11
13
15
17
19
-1
-3
Hours in light


Figure 5a-d.


6
Cyclotella meneghiniana
TQ
a
16:8 20°C
b
16:8 15°C
c
20:4 20°C
d
20:4 15°C
Percent
Hours in light
1
3
5
7
9
11
13
15
17
19
-1
-3
0
20
40
60
80
100

Figures 6a-d.

## LIST OF FIGURES

# PHOTOPERIOD AND TEMPERATURE EFFECTS ON DIATOM LIPID COMPOSITION

## II. *MELOSIRA VARIANS*


## ABSTRACT

*Melosira varians* has a world wide distribution and tolerates a wide range of environmental parameters as a littoral taxon of eutrophic waters. Previous studies in our lab have demonstrated that although *M. varians* will grow under a variety of temperatures and light regimes, it grows best under short-day conditions. Analyses of the diel patterns of lipid composition of this diatom grown under light/dark regimes ranging from 12:12 h to 20:4 h L/D with temperatures between 15 and 20°C demonstrates that polar lipid production is highest under the 12:12 h L/D regime. However, with the exception of the regime 16:8 h L/D at 20°C, the differences in lipid composition, for the most part, are not statistically significant. Diel lipid composition patterns will be discussed in terms of the known distribution records of this diatom.


## INTRODUCTION

Although *Cyclotella meneghiniana* and *Stephanodiscus binderanus* demonstrate daylength preferences for growth, we found that *Melosira varians* grew under a wide variety of temperature and light regimes. We have extended these studies and present data which demonstrate that lipid content is less variable under a variety of growth conditions. Trends in lipid class composition suggest that species specific responses aid in understanding the distributional ecology of this diatom in nature.

## MATERIALS AND METHODS

For growth curve studies, algae between 2 and 3 months old (in lag phase growth) were inoculated into fresh WC medium (Guillard 1975) and placed on a shaker table in a walk-in growth chamber which is set at a temperature and light/dark cycle dependent upon the experiment. Prior to introduction of the algae into the new medium with different growth conditions, the batch cultures were acclimated to the temperature and light regime for a period of two weeks to ensure that results were not those of temperature or light shock. Aliquots were withdrawn in duplicate from thoroughly mixed 6 liter flasks 2-3 times a week for a period of approximately 6 weeks. Depending on cell density, between 75 and 200 mL were withdrawn per sample, filtered onto prewashed and preweighed Gelman A/E glass fiber filters, air dried, then dried in a vacuum at 60° for 24 hours. The filters were then reweighed to determine dry weight, and frozen for subsequent lipid extraction. Concurrent with dry weight analyses, smaller volumes of culture (9 mL) were withdrawn and placed in a tube containing paraformaldehyde-glutaraldehyde at final concentrations of 1% in 0.05 M sodium cacodylate buffer. Cell counts were performed on these samples with either a hemocytometer or plankton counting cell to determine cell densities.

For lipid analysis, the frozen filters were placed in a pre-extracted thimble and extracted in a micro-Soxhlet with chloroform for 12 hours (Orcutt and Patterson 1975). The extract was then concentrated in a Kuderna-Danish flask, evaporated under nitrogen, redissolved in chloroform and separated with a separatory funnel. This extract was dried under nitrogen stream in pre-weighed Teflon lined screw cap amber vials and weighed for total gravimetric lipid. Samples were then flushed under nitrogen and frozen for analyses of the lipid classes.

For lipid class analysis, samples were redissolved in methylene chloride to concentrations of 20 to 50 μg lipid in spotting volumes of 10 to 20 μL. The samples were spotted with Hamilton syringes onto cleaned and blank scanned silica coated chromarods (type SIII), held in a frame and developed and scanned in an Iatroscan Mark IV (TLC-FID, FTID) system using a development system described by Parrish (1986).

The development is three staged, consisting of the following:

1. Developed in 50 mL solution of hexane, diethyl ether, and formic acid (98:2:0.05) for 25 minutes, conditioned for 5 minutes, and redeveloped in the same solvent system for 20 minutes. Rods are then partially scanned for hydrocarbons, wax esters, and ketones.
2. The rods are then reconditioned and developed in 50 mL of a solution of hexane, diethyl ether, and formic acid (80:20:0.1) for 50 minutes. The rods are then again partially scanned for triglycerides, FFA alcohols, and sterols.
3. The third development consists of two 15 minute developments in 100% acetone, followed by two 10 minute developments in 50 mL of solution containing dichloromethane, methanol, and water (5:4:1). During the last scan, the FTID detector is also used for the additional detection of N in Chla and phospholipids. The rods are scanned their entire length for chlorophyll *a*, acetone-mobile polar lipids, and phospholipids.

Quantitative determinations of lipid class composition are based on dose-response calibration equations generated by analysis of a wide range of concentrations of standards for each lipid class.

## RESULTS

Cell counts and percent total extractable lipid (TEL) for growth regimes tested are presented in Figures 1-4. Increases in cell numbers were observed when cells were incubated at either 16:8h L/D 20°C or 12:12 h L/D 20°C. Cell numbers were maintained or reduced under other conditions.

The 24 hour averages for all lipid classes are presented in Table 1. The lowest neutral/polar ratios were observed when cells were grown under a 12:12h L/D 20°C or 20:4h L/D 15°C regime. Under all regimes examined, however, the neutral/polar lipid ratio observed was higher than that reported for *Cyclotella*. Lipid class composition was fairly uniform across all regimes, with few statistically significant differences.

Twenty-four hour profiles of selected lipid classes and ratios are presented in Figures 5-7. The data are plotted as a function of time in the light. With the exception of the 16:8 (15°C) profile, there is a general decrease in the neutral/polar ratio (Fig. 4) at approximately 11 hours after the lights go on, followed by an increase. However, the pattern appears to be related more to absolute time of day than to the hour of light or dark exposure.

Chlorophyll values (Fig. 6) generally increase throughout the day. Triacylglycerol values remain fairly constant, with some reduction in the latter half of the light cycle.

## DISCUSSION

The cell count data suggest that *Melosira varians* is able to either grow or maintain cell numbers when cultured under a variety of light regimes. However, it appears to have a preference for either short or very long days. The lipid class composition data reveals that lipid profiles are rather uniform throughout the day under most conditions. If percent total neutral lipid is interpreted as a measure of stress, then growth under the 16:8h L/D regimes is the most stressful, although increases in cell numbers are observed in this regime at 20°C.

Our data, then, suggest that *Melosira varians* should be distributed around the autumnal and vernal equinox at mid latitudes. A reexamination of the data of Stoermer and Yang (1969) reveals that occurrences of *M. varians* in Lake Michigan were indeed clustered in the periods March-May and September-October, with no occurrences reported in the months June-August, when the light dark cycle most closely approximates 16:8h L/D at 45° latitude. Our data further suggest that this species would grow well at higher latitudes where longer (i.e., 20:4h L/D) light periods are encountered.

*Melosira varians* is characterized as a cosmopolitan taxon which tolerates a broad range of ecological conditions. A cursory survey of diatom literature suggests a distributional pattern of non-existent at equatorial latitudes (Foged 1976, Hustedt 1938), and present at mid latitudes 30-45°N (Foged 1959, Stoermer and Yang 1969). It is rare at the northern European latitudes, 55N° (Foged 1954), and common at moderately high latitudes, 64-66°N (Foged 1974). It is rare (68°N, Foged 1981) to absent (>70°N) at high latitudes (Patrick and Freese 1961, Foged 1964).

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## ACKNOWLEDGMENTS


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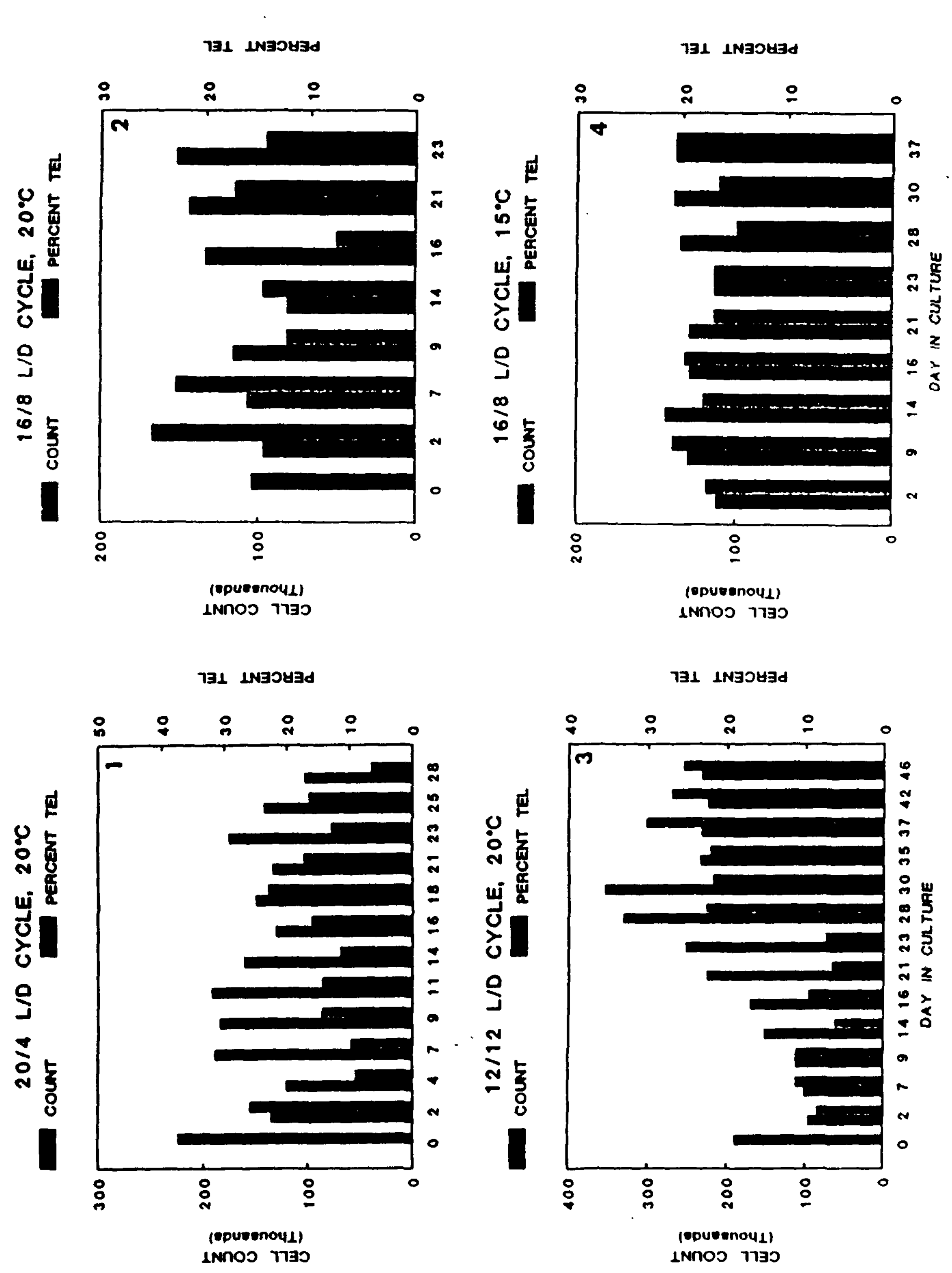
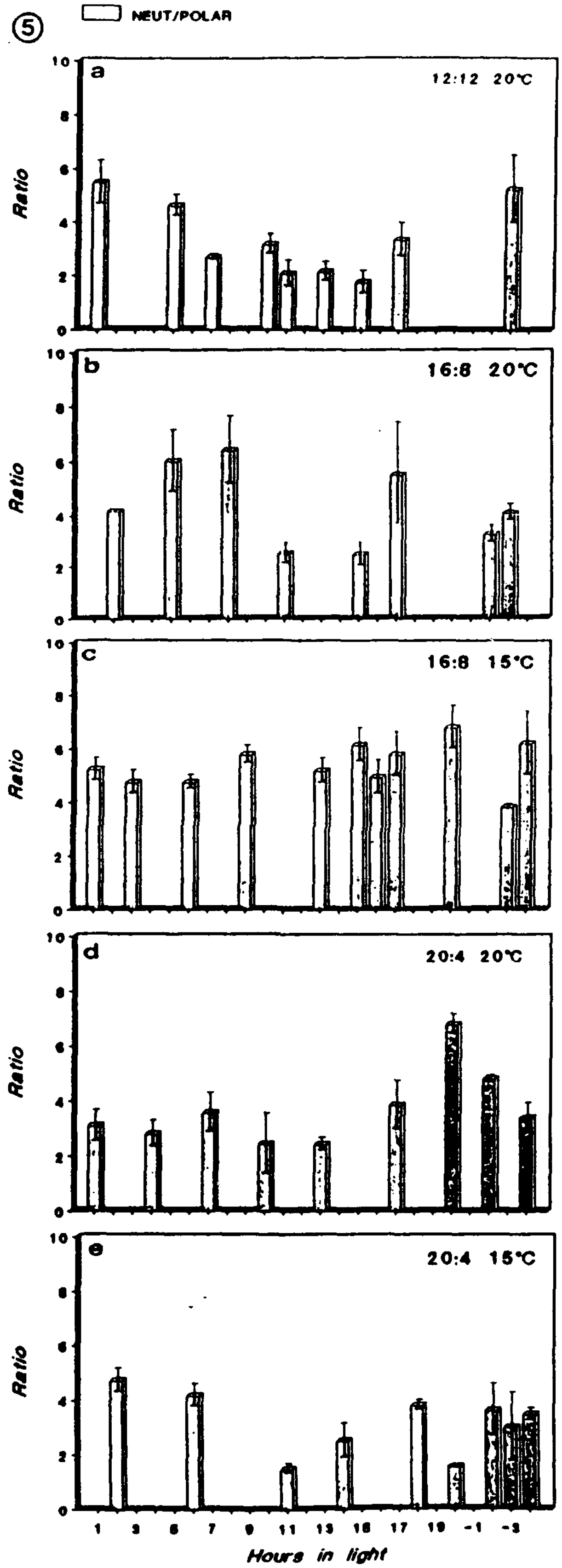


20/4 L/D CYCLE, 20°C
COUNT
PERCENT TEL
CELL COUNT (Thousands)
PERCENT TEL
1
16/8 L/D CYCLE, 20°C
COUNT
PERCENT TEL
CELL COUNT (Thousands)
PERCENT TEL
2
12/12 L/D CYCLE, 20°C
COUNT
PERCENT TEL
CELL COUNT (Thousands)
PERCENT TEL
DAY IN CULTURE
3
16/8 L/D CYCLE, 15°C
COUNT
PERCENT TEL
CELL COUNT (Thousands)
PERCENT TEL
DAY IN CULTURE
4


Figures 1-4.



5
NEUT/POLAR
a
12:12 20°C
b
16:8 20°C
c
16:8 15°C
d
20:4 20°C
e
20:4 15°C
Ratio
Hours in light


Figure 5a-e.

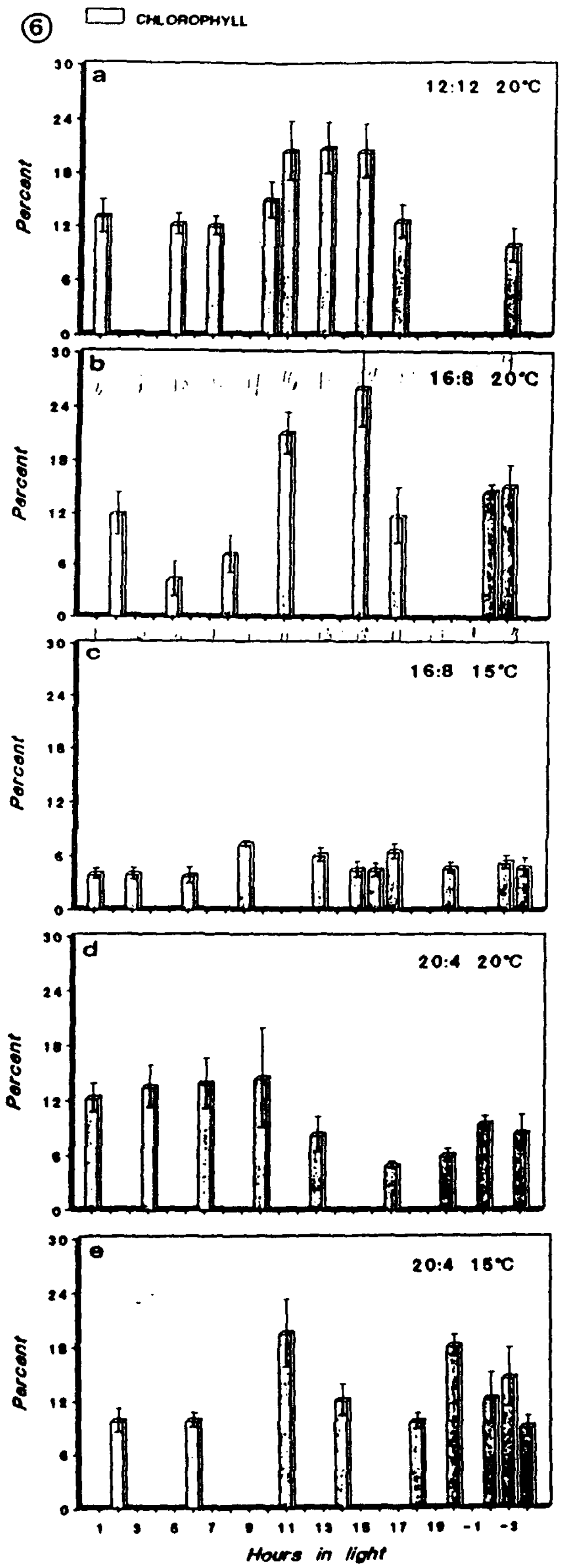
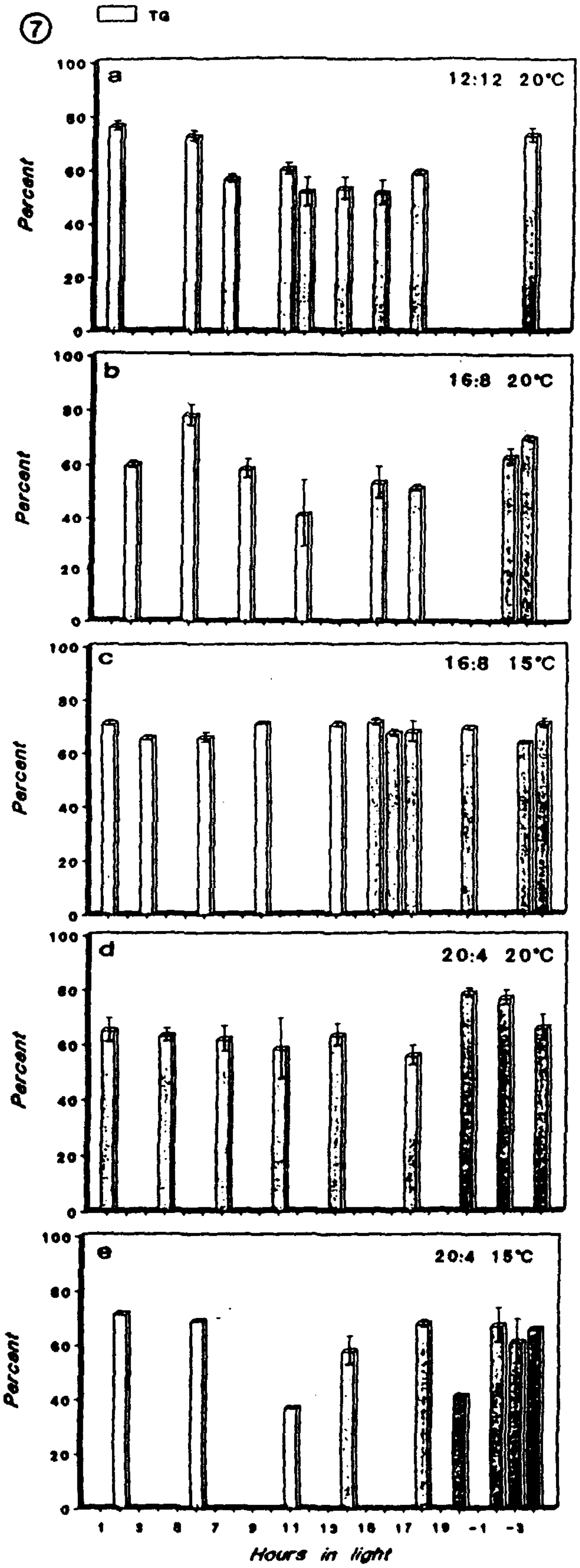

6
CHLOROPHYLL
a
12:12 20°C
b
16:8 20°C
c
16:8 15°C
d
20:4 20°C
e
20:4 15°C
Percent
0
6
12
18
24
30
1
3
5
7
9
11
13
15
17
19
-1
-3
Hours in light

Figure 6a-e.


7
TG
a
12:12 20°C
b
16:8 20°C
c
16:8 15°C
d
20:4 20°C
e
20:4 15°C
Percent
Hours in light

Figures 7a-e.

## LIST OF FIGURES

## Status of Unpublished Data

*Section C – Unpublished Data Status*

Aside from data which has been presented or published, data analysis and writing is still continuing on the following studies:

1. Flow cytometric studies

   Flow cytometric studies were initiated in conjunction with the Undergraduate and Summer Research Opportunity Programs for minority students through the University. Ms. Annabelle Javier worked on a project to assess the relationship between assessments of neutral lipid content determined flow cytometrically with Nite Red staining and values for neutral lipid classes determined via the Iatroscan FID methodology we have been using since the start of the project. Ms. Javier is interested in continuing her studies so that she can publish a paper in a scientific journal and has reapplied for a summer grant through the University program. So we expect that a publication will result from this combined effort in early 1993.

2. Natural phytoplankton assemblage studies

   Last year, through a grant from the University, we were awarded ship time for preliminary studies of the effect of chlorinated benzenes on natural phytoplankton assemblages. We utilized this time to determine if natural assemblages had diel periodicities on lipid content and composition, and to determine if exposures initiated at different times of day resulted in different responses. Iatroscan studies of this experiment are complete and the raw data is presented in Appendix 1. Preliminary results suggest that over a 24 hour period, significant differences were observed in exposures initiated at 0400 hrs compared with exposures initiated at 1600 hrs. There was also a ca. 50% drop in dry weight in 24 hrs. Permanent phytoplankton slides were made of these experiments, but data analysis on phytoplankton composition and abundance is still incomplete. A more complete assessment will be made this summer when Dr. Andresen presents the results at the 12th International Symposium on living and fossil diatoms.

3. Diel lipid composition studies

   Data analyses for diel lipid composition studies are complete and have been presented at Scientific Meetings. These data will be used for the publication of three additional manuscripts.

## Conclusions

Our results may be summarized as follows:

1. Diel lipid content appears to be more variable than variation occurring during a growth cycle. Freshwater diatoms apparently differ from marine diatoms in that triacylglycerol production is reduced before the onset of rapid growth and this production resumes even while cells are growing logarithmically.

2. Diel variation in lipid content varies with the diatom species tested.

3. We have demonstrated that some diatoms appear to have strong daylength preferences. Lipid content varies with daylength.

4. Sensitivity of diatoms to chlorinated benzenes appears to be related to more lipid composition than total extractable lipid, in short-term (i.e., 24 hour to 10 day) exposure experiments. We have demonstrated that the time of initial exposure to the chlorinated benzene determines the toxicity of the compound in dose ranges near water solubility. The effect is repeatable and appears to be related to the lipid composition, specifically polar lipids and chlorophyll content.

## Recommendations

1. Studies should be initiated with other algal divisions to assess diel lipid composition variability.

   The extension of these studies to standard bioassay organisms such as *Selenastrum* is critical to determine if timing of exposure affects these standard bioassays.

2. Although at low concentrations the chlorinated benzenes as a rule did not appear to be extremely toxic, the effects in assemblages were not studied thoroughly. The real test in ecosystem studies of toxicants is to determine secondary effects and we strongly urge the development of competition experiments to determine if viability is reduced.

## Appendix 1

Summary of exposure of lake phytoplankton assemblages to 1,2,3-trichlorobenzene for 24 hrs. One exposure was initiated at 0400 h; the second exposure was initiated at 1600 h.

| Sample Description | Sample | Relative Percent Composition TEL HC | WE/SE | TG/FFA | ALC | ST | Chl *a* | Ampl | PL | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| 11 July | 0400 30M | 78.53 | 1.10 | 7.01 | 0.67 | 1.53 | 2.13 | 2.23 | 6.81 | 100 |
| Lake | | 2.16 | 0.41 | 1.71 | 0.37 | 0.31 | 0.21 | 0.32 | 1.00 | |
| Assemblage | | | | | | | | | | |
| 12 July | 0400 30M | 73.65 | 1.07 | 14.01 | 0.99 | 2.24 | 3.13 | 1.14 | 3.77 | 100 |
| Lake | | 0.24 | 0.32 | 2.93 | 0.19 | 0.03 | 2.04 | 0.16 | 1.40 | |
| Assemblage | | | | | | | | | | |
| 12 July | 0400 A | 42.67 | 1.56 | 34.99 | 0.60 | 5.36 | 5.35 | 1.26 | 8.22 | 100 |
| Control | 0400 B | 1.99 | 0.10 | 2.92 | 0.33 | 0.35 | 0.74 | 0.22 | 1.10 | |
| Bottle | | | | | | | | | | |
| 12 July | 0400 A | 47.08 | 1.12 | 36.69 | 0.77 | 4.41 | 3.66 | 1.60 | 4.67 | 100 |
| Exposed | 0400 B | 2.21 | 0.07 | 3.70 | 0.30 | 0.14 | 0.34 | 0.43 | 1.30 | |
| Bottle | | | | | | | | | | |
| 11 July | 1600 30M | 49.36 | 3.55 | 30.34 | 0.44 | 4.25 | 4.34 | 1.14 | 6.58 | 100 |
| Lake | | 3.31 | 0.64 | 4.56 | 0.59 | 0.33 | 0.31 | 0.56 | 1.27 | |
| Assemblage | | | | | | | | | | |
| 12 July | 1600 A | 66.80 | 1.75 | 16.07 | 0.82 | 3.64 | 5.88 | 0.38 | 4.67 | 100 |
| Control | | 0.24 | 0.11 | 2.36 | 0.44 | 0.19 | 1.53 | 0.11 | 0.05 | |
| Bottle | | | | | | | | | | |
| 12 July | 1600 A | 72.03 | 1.01 | 11.46 | 1.07 | 3.90 | 4.78 | 0.74 | 5.01 | 100 |
| Exposed | | 2.37 | 0.20 | 1.06 | 0.15 | 0.08 | 0.77 | 0.02 | 0.28 | |
| Bottle | | | | | | | | | | |

## Appendix 2

Summary of exposure of lake phytoplankton assemblages to 1,2,4-trichlorobenzene for 24 hrs. One exposure was initiated at 0400 h; the second exposure was initiated at 1600 h.

| Sample Description | Sample | HC | WE/SE | TG/FFA | ALC | ST | Chl *a* | Ampl | PL | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Relative Percent Composition TEL | | | | | | | | |
| 11 July Control Bottle | 0400 A | 32.33 | 1.68 | 31.48 | 0.91 | 3.83 | 22.44 | 0.56 | 6.77 | 100 |
| | 0400 B | 2.16 | 0.28 | 4.49 | 0.27 | 0.65 | 9.40 | 0.34 | 1.44 | |
| 12 July Exposed Bottle | 0400 A | 31.93 | 5.03 | 44.24 | 0.57 | 5.19 | 5.21 | 1.22 | 6.61 | 100 |
| | 0400 B | 1.17 | 2.14 | 1.11 | 0.32 | 0.25 | 1.08 | 0.20 | 0.26 | |
| 11 July Control Bottle | 1600 A | 70.24 | 0.51 | 12.67 | 0.97 | 3.66 | 4.61 | 0.96 | 6.40 | 100 |
| | | 0.69 | 0.02 | 2.75 | 0.25 | 0.08 | 0.12 | 0.28 | 1.31 | |
| 12 July Exposed Bottle | 1600 A | 66.23 | 0.43 | 14.64 | 1.26 | 3.80 | 3.58 | 1.27 | 8.80 | 100 |
| | | 0.63 | 0.01 | 2.21 | 0.01 | 0.15 | 0.13 | 0.29 | 1.33 | |